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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	13
Reportable Outcomes.....	14
Conclusions.....	14
References.....	14
Appendices.....	attached

INTRODUCTION

Hyperploidy is a well-known pathological hallmark of cancer cells including prostate cancer. The mechanisms underlying this form of genomic instability are essentially unknown. We have identified a pathway in fission yeast critical for maintaining DNA replication control. Two genes in the pathway, POP1 and POP2, encode WD40 domain proteins that heterodimerize to bind to regulatory proteins and promote their ubiquitin-dependent proteolysis. The targets of this proteolytic destruction machinery include regulators of cyclin-dependent kinases, cyclins, Cdk inhibitors, and the replication initiator protein, Cdc18. Mutations in Pop1 or Pop2 cause yeast to accumulate these regulators and to prominently overreplicate their genomes ($>8N$). Accordingly, the POP genes behave as “rereplication suppressors” in yeast. We have identified human and mouse homologs of the POP genes which we have called hPOP1 and mPOP1. These genes encode proteins that are overall 21% identical and 55% similar to yeast equivalents, but have higher degrees of homology in some domains. We have begun to test whether these proteins serve a similar role in mammalian cells. Using radiation hybrid mapping and FISH, we have mapped the human POP1 to a region that has been suggested to contain a potential tumor suppressor for urogenital cancer.

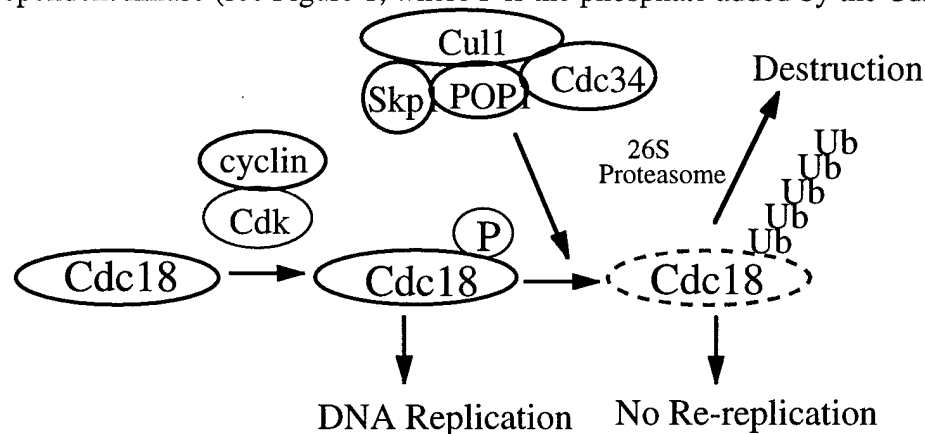
BODY:

BACKGROUND

Rereplication control may be important for tumor suppression: Genome integrity is surveyed by “checkpoints”, which are control mechanisms that survey the state of the DNA and ensure the completion of cell cycle events. In a normal somatic cell cycle, for example, complete replication of the genome in S phase is always followed by sister chromatid separation and cell division in mitosis. A related, but formally distinct mechanism ensures that each segment of the genome is replicated only once in each S phase (1) This is termed re-replication control.

Kinzler and Vogelstein (2) suggest an expanded definition of tumor progression, wherein mutations in “caretaker” genes, like DNA repair enzymes, do not directly promote tumor growth, but instead promote an increased mutation rate. Examples include mutations in DNA mismatch repair enzymes in hereditary nonpolyposis colorectal cancer and mutations in nucleotide excision repair in Xeroderma pigmentosum. Once a caretaker mechanism is defective, mutations in checkpoint genes including p53 contribute an additional level of genomic instability, favoring mutations in oncogenes. We suggest that re-replication control make be an essential “caretaker”.

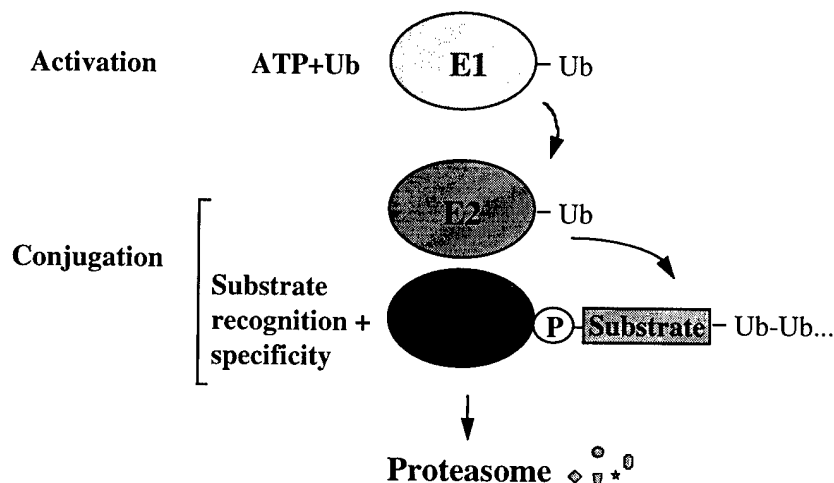
Rereplication control requires ubiquitin-dependent destruction of a replication initiator: Recent studies suggest that re-replication is controlled at least in part through initiation of DNA replication. DNA replication initiates at discrete sites within the genome, termed replication origins. Two sets of factors are needed for the initiation of DNA replication. The first set includes the Origin Recognition Complex (ORC), a complex of proteins that bind to origins, and Cdc18, a critical initiator protein conserved from yeast to man (3). Binding of this “pre-replicative complex” to the chromatin in G1 is believed to set-up replication. At the transition to S phase, a second set of factors including cyclin-dependent kinases (Cdks) trigger replication. Cdc18 is a direct target of the cyclin-dependent kinase (see Figure 1, where P is the phosphate added by the Cdk).



The POP complex blocks rereplication by destroying the Cdc18 replication initiator

After initiation of DNA replication, the phosphorylated Cdc18 is thought to be directly bound by the POP1 protein (4-6). This binding recruits a ubiquitination enzyme complex (including POP1, and fission yeast homologs of proteins called Skp1, Cul1, and Cdc34). This complex then promotes poly-ubiquitination (depicted as a chain of Ubs) and destruction of Cdc18 (depicted by the dotted line), thereby blocking re-replication (see Figure). Our studies in fission yeast have demonstrated that the Cdk-dependent proteolysis of Cdc18p during S phase, is central to the inactivation of a fired origin.

Ubiquitination basics and remaining questions: The formation of ubiquitin-protein conjugates requires the ordered action of three enzymes.



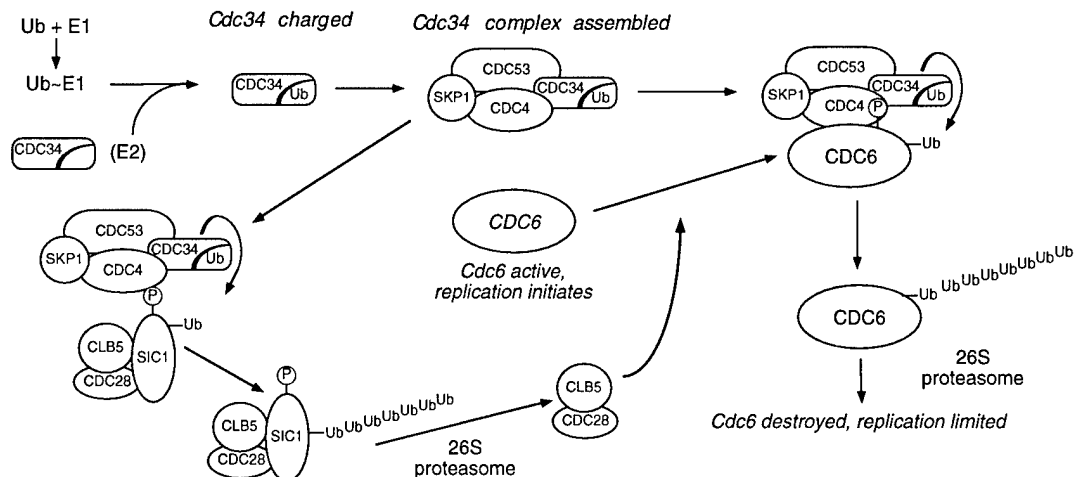
Conserved elements of ubiquitin-mediated proteolysis systems

An ubiquitin-activating enzyme, E1, uses ATP to form a thiolester bond between ubiquitin and itself (see Figure). E1 then transfers ubiquitin to an ubiquitin-conjugating enzyme, E2. Lastly, ubiquitin is transferred to a substrate directly by the E2 or by an E2 acting together with a ubiquitin-protein ligase, or E3. While some proteins are posttranslationally mono-ubiquitinated for other apparent regulatory functions (non-catabolic ubiquitination), proteolytic destruction by the 26S proteasome requires the formation of poly-ubiquitin chains, typically 4-8 ubiquitins long, which apparently form the signal for transport into the proteasome. The mechanisms of E3-mediated ubiquitin addition and how poly-ubiquitin chains are formed are poorly understood.

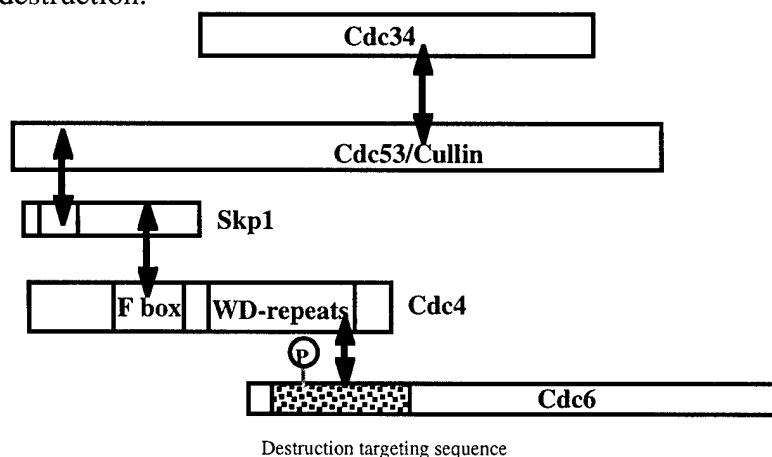
Ubiquitination of replication proteins and cyclin-dependent kinase regulators

requires a complex containing POP/Cdc4 proteins: The mechanism for proteolytic destruction of Cdc18p requires a ubiquitination ligase complex containing the ubiquitin conjugating (or E2) enzyme Cdc34 to promote the addition of chains of the small, highly conserved protein called ubiquitin. Poly-ubiquitin chains target proteins for degradation by the 26S proteasome (see the attached recent reviews by Jackson and Wolf and Jackson). In fission yeast, this complex also contains two related proteins called Pop1p and Pop2p, which recognize and bind to Cdc18p to recruit the ubiquitination enzymes. Interestingly, strains deleted for *pop1* and *pop2* show increased levels of Cdc18p and a high level of re-replication.

In the yeast *S. cerevisiae*, a similar complex of proteins including Cdc4p, a homolog of the Pop proteins, Skp1, the Cul1 homolog Cdc53, and Cdc34 function to allow the entry into S phase by destroying the Sic1 inhibitor of the cyclin-dependent kinases that drive S phase (7-10).



Recent studies have elucidated part of the architecture of the SCF (see Figure, next page) and a reconstitution of the ubiquitin ligase activity from a limited number of purified components has helped define some of the functions of this complex (7-10). Substrate recognition appears to be mediated by the POP or Cdc4 proteins through their C-terminal WD40 domains. Similar WD40 domains are found in numerous proteins and are believed to mediate protein-protein associations. The Pop proteins and Cdc4 also contain a short protein motif called an F-box. The F-box is required for the binding of the Skp1 protein. Skp1, in turn, recruits Cul1 in human cells or its homolog Cdc53p in yeast cells. Cul1 is an example of a new family of at least five proteins called cullins. The cullins may form scaffolds upon which SCF ubiquitin ligases are assembled. Together the F-box protein, Skp1, and the cullin can recruit the Cdc34 ubiquitin conjugating enzyme and also bind the substrate, thereby allowing transfer of ubiquitin chains to the substrate and ultimately destruction.



In addition to the POP proteins, a large number of proteins contain F-boxes. Julie Regain (see Key Personnel) in my laboratory has used Skp1 in a genetic interaction ("two-hybrid screen") and has identified over a dozen novel F-box proteins, which appear

to be adapters for distinct target proteins. Accordingly, we suspect that a large number of cellular regulators may be targeted for proteolytic destruction by variants of the SCF ubiquitin ligase. Does it seem likely that each protein will then have its own adapter?

Dieter Wolf in my laboratory demonstrated that Pop1 and Pop2 must form a hetero-oligomer to bind and target the destruction of the Cdc18 replication protein in fission yeast. We mapped an N-terminal interaction domain between Pop1 and Pop2 and found that Pop2 variants with mutations in the Pop1 interaction domain failed to bind Pop1, and failed to bind, ubiquitinate, and mediate destruction of their substrate, Cdc18. Thus, it may be in general that distinct F-box proteins may interact to bind substrate and it may be that combinations of a smaller number of F-box proteins may bind to a larger number of substrates. A further interesting possibility is that by forming higher order complexes, the SCF may contain multiple binding sites for assembling ubiquitin subunits, a structural possibility that could explain how ubiquitin chains are processively assembled.

SCF ubiquitin ligases are involved in growth control in many organisms

In the worm *C. elegans*, mutations of a homolog of Pop1 or of the cullin Cul1 cause hyperplasia of many tissues. Accordingly, there is a suggestion that this pathway also mediates growth control. Indeed, the stability of different G1 cyclin-dependent kinases and Cdk inhibitors are now being shown to be regulated by distinct SCF complexes (11,12). Because these diversely deployed regulatory ubiquitin ligases are important for growth control, there is a strong possibility that they may be important in human cancer. These studies will begin to address these hypotheses in breast cancer and other tumors.

RESULTS

In the initial review of the proposal, several of the projects proposed in the original work plan were suggested to be changed. Indeed, the overall focus of the project has changed considerably, so the original work plans have been substantially modified.

Project A: Construction of transgenic mice expressing Cdc18 and ORC1 dominant negatives

This project was removed from the emended work plan and was not further pursued.

Project B: Construction of POP1 knock-out strains

This project is still of interest, but the funding provided was not adequate to undertake this project.

Project C: Genetic studies of a human POP1 homolog

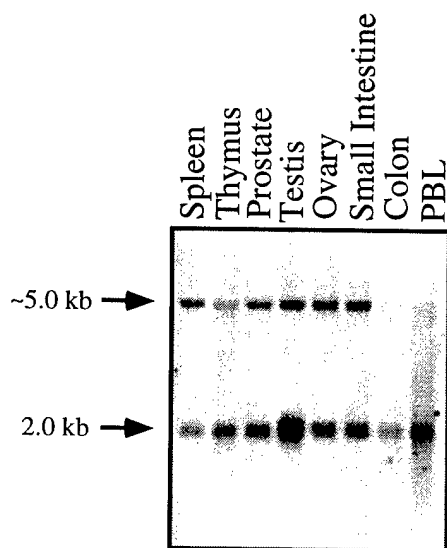
This project became the major focus of the grant. **We succeeded by identifying that the human POP1 as tightly linked to a tumor suppressor for bladder cancer on chromosome 9 (9q34.1).** The original work plan included these aims, now completed.

Task	Description	Statu
1	Clone and sequence full length versions of the human POP1 genes	Complete
2	Clone a bacmid for human POP1.	Complete
3	Perform Fluorescence In Situ Hybridization and radiation hybrid analyses with POP1 to map the locus of the gene	Complete

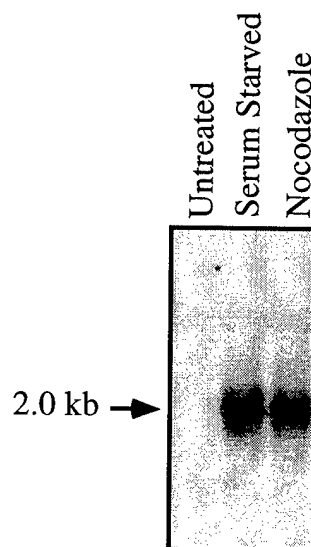
In addition to these planned tasks, we used the information about the potential role of hPOP1 in bladder cancer to further explore hPOP1. Expression studies supported that hPOP1 is overexpressed in 2 of 4 bladder tumors. Other functional studies have been attempted and some studies are ongoing.

Results & Data:

A. We have cloned human and mouse homologs of the POP proteins: Using both a two hybrid screen with the Skp1 component of the SCF complex and identifying ESTs by homology search, we have found a human homolog of the POP genes. We have also isolated a mouse homolog. We have named these genes hPOP1 and mPOP1. hPOP1 is expressed as a 2.0 kb message in many tissues. The message level is low in asynchronous cells and in G1, but accumulates in S, G2, and M phases and in quiescent cells (see Figure and data not shown).



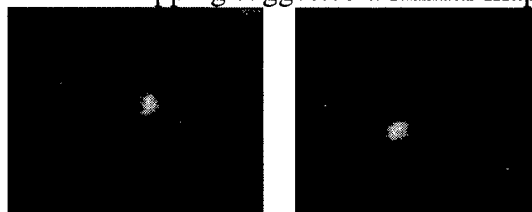
Northern blot of RNA from human tissues



Northern blot of treated NIH 3T3 cells

We have raised antibodies to hPOP1 and found that hPOP1 encodes a protein of approximately 75 kilodaltons. Coimmunoprecipitation studies show that hPOP1 is associated with the human Skp1, Cul1, and Cdc34 proteins, supporting the physical presence of an SCF^{hPOP1} (discussed below). The association of hPOP1 with these other SCF components supports our model that this protein functions similarly to the complex in fission yeast. We are now beginning a series of studies to biochemically test these ideas further, although those studies are outside the scope of this grant.

B. Genetic mapping studies of the human POP1 protein suggests linkage to a tumor suppressor for bladder cancer: We used our cDNA for hPOP1 to isolate a bacterial artificial chromosome (BAC) for fluorescence in situ hybridization (FISH) and to design a PCR assay for the presence of hPOP1 gene in genomic DNA. Using our PCR assay, we tested a radiation hybrid (RH) panel as an independent means of mapping the hPOP1 gene. Both FISH and the RH mapping suggested a similar map location at 9q34.



Fluorescence In-situ Hybridization localizes
hPOP1 to human chromosome 9q34

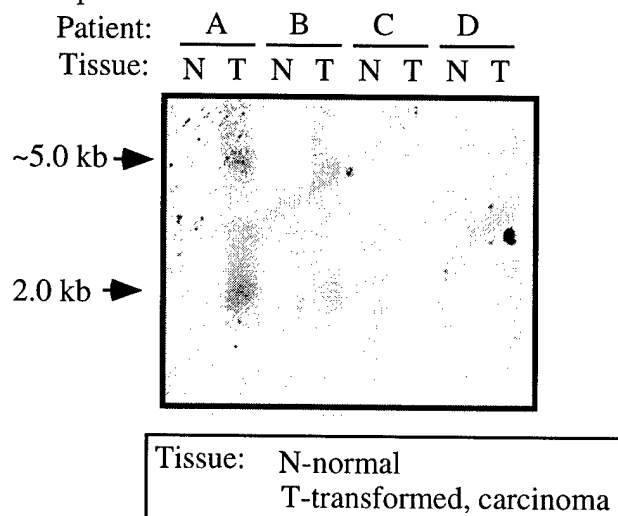
Literature and database searches indicated a small number of human disease loci were in this region (see <http://www.gene.ucl.ac.uk/chr9/>). We have been able to eliminate several and have focused on a potential tumor suppressor for bladder cancer (13, 14). LOH studies in human tumors support the loss of this locus as an initiating step in specific forms of bladder cancer (see Sidransky in Kinzler and Vogelstein, 1997).

C. Genomic structure of hPOP1

Using oligonucleotide sequencing primers based on the hPOP1 sequence, we have been able to sequence our BAC to define the exons and the structure of the chromosomal region around hPOP1. At this point, we have completely identified 5 C-terminal exons and portions of their intervening introns. Our BAC did not include the 5' most sequence and we have cloned a second BAC that now contains that information. We should be able to finish the genomic structure by sequencing portions of the second BAC. These studies will allow us to use PCR to clone the coding regions of hPOP1 from genomic DNA, which in many cases will be less difficult than isolating RNA and performing

reverse transcription and PCR amplification (RT-PCR cloning). The ability to clone and sequence hPOP1 from small amounts of DNA will simplify the identification of point mutations and other alterations to the hPOP1 gene in tumor samples.

D. hPOP1 is overexpressed in bladder tumors: As a first way of looking at whether the hPOP1 gene might be altered in bladder tumors, we performed Northern blot analysis on RNA from four patients, comparing matched tumor and normal tissue. This analysis revealed a large increase in the level of hPOP1 expression in the tumor tissue from two of the patients. We will now expand that analysis to look at more tumors and also to begin to analyze whether the increase in expression corresponds to an increase in the levels of hPOP1 protein or a lack of hPOP1 protein. For oncogenes, the overexpression of a gene is often an important mechanism for tumorigenesis because increased levels of protein and activity will create a positive signal for cell proliferation. However, for some tumor suppressors, which may be how POP1 functions, the overexpression of the tumor suppressor mRNA would have a different meaning. In the case of the p16INKa tumor suppressor for melanoma, the gene is often overexpressed in tumors. This reflects that fact that the existing allele(s) of the gene do not encode a functional protein. To compensate homeostatically, the cell has a mechanism that presumably senses the absence of the protein and tries to compensate by increasing the level of the transcript. However, if the protein is defective or absent, increasing the expression of the mRNA encoding it does not restore activity. Thus, the increased message level is a reflection of a futile attempt to restore protein levels.



In the case of the POP genes in fission yeast, we are already aware of the presence of such a mechanism (D. Wolf and P. Jackson, unpublished work). The transcriptional circuitry that controls the expression of the POP genes is sensitive to the level of POP activity. In this case, we know that the stability and thus possibly the activity of a critical

transcription factor, called Res1, is controlled by the POP proteins themselves. Thus, our prediction is that some of the tumors that have highly overexpressed hPOP1 mRNA may actually have inactivating mutations in the protein. Accordingly, it may be very productive to first screen for RNA alterations and then sequence the hPOP1 gene from those tumors or cell lines.

To be able to look at the hPOP1 protein, we have raised antiserum to bacterially expressed protein in rabbits and mice and affinity purified these antibodies. The purified antibodies detect a 75 kilodalton protein (the expected size) on Western blots and show only one additional species, which may either be a modified form, a related protein, or a cross-reacting band. Competition experiments suggest that both forms are specific.

To test whether the hPOP1 protein exists in a complex with the other human SCF components Cdc34, Cul1, and Skp1, we performed coimmunoprecipitation experiments. Using extracts from human kidney endothelial cells or HeLa cells, we have used antibodies against hPOP1 to pull down an associated complex. Western blots of the immunoprecipitates reveal comparable amounts of the human Skp1 and Cul1 proteins, and slightly reduced amounts of Cdc34 (we have prepared affinity purified antibodies that recognize each of these proteins). Additionally, we have found that substantial amounts of the endogenous human Cdc6 protein are present in these immunoprecipitates. We have also used cotransfection studies to express epitope-tagged variants of these proteins in mammalian cells and have reconstituted several of these interactions in cells. In sum, we believe that the hPOP1 protein is an functional ortholog of the POP proteins in yeast.

We have also begun to use these antibodies to examine the subcellular and tissue localization of the hPOP1 protein. Preliminary immunofluorescence data has shown that the majority of the hPOP1 protein is nuclear, but that some cells show larger amounts of cytoplasmic staining. In fission yeast, we have some evidence that the Pop proteins may shuttle between nucleus and cytoplasm (D. Wolf and P. Jackson, unpublished), so again our findings there may inform what happens in human cells. In collaboration with Dr. Matt van de Rijn, a talented surgical pathologist here in the Department of Pathology at Stanford, we have begun to use our antibodies to examine the localization of hPOP1 in formalin-fixed tissue from bladder carcinoma and surrounding normal tissue. Thankfully, our antibodies specifically stain the formalin samples, thus affording us the possibility of examining a large repository of such materials. Our preliminary evidence shows a highly increased nuclear staining with the hPOP1 antibodies in the tumor tissue and a balance of nuclear and cytoplasmic staining in surrounding tissue. We need additional studies to support these findings, but our first studies look very promising.

E. A Two Hybrid Screen with hPOP1. To identify interacting proteins with human Pop1, we performed a two-hybrid screen with the human Pop1 protein, screening a human HeLa cell library for interacting proteins. This screen did not clearly identify any interesting candidate interacting proteins. To pursue this further, the screen would need to be repeated with possibly different baits or libraries.

F. A successful screen for additional F-boxes.. As noted before, we cloned and collected a large number (~45) novel F-box proteins. These form a set of tools to begin a comprehensive examination of the role of ubiquitin ligases in cancer. At this time, those are classified on the basis of whether they are F-box proteins containing a WD40 domain (called Fbw), a leucine-rich repeat domain (called Fbl), or are one of a catch-all class missing either of these motifs (Fbx). We are currently collecting the "complete" human set of cDNAs (not necessarily full length). At this time, there are five Fbw, 13 Fbl, and 28 Fbx proteins identified. These results were recently published in *Current Biology*.

Reference: Regan-Reimann JD; Duong QV; Jackson PK. (1999).

Identification of novel F-box proteins in *Xenopus laevis*. *Current Biology* 9, R762-3.

We intend to use these clones to design a set of PCR primers for quantitative PCR ("Taqman"), which will allow a much more reliable determination of RNA abundance for these physiologically critical genes. First, we will determine the levels of F-box proteins in normal versus the 62 transformed cell lines obtained from the Developmental Therapeutics Program (DTP). Next, we will determine the effect of critical physiological alterations (changes in media, growth factors, DNA damage, microtubule or actin poisons, cancer chemotherapeutic drugs) in normal versus transformed cells to determine whether the response of any of these F-box proteins is clearly altered in transformed cells. These correlations may be critical for understanding transformed cell biology and the response of specific tumor types to physiological changes and chemotherapy.

KEY RESEARCH ACCOMPLISHMENTS

- Full length cDNA clones of human and mouse hPOP1 obtained and sequenced
- Expression of a protein of the appropriate size indicates the human POP1 is a functional gene
- A genomic clone of human POP1 was obtained and sequenced. A map of the genomic structure was obtained (See Figure 2).
- Antisera against hPOP1 were obtained and a 46 and 65 kDa species identified

- Expression studies indicate that the hPOP1 gene is expressed in many tissues including bladder
- hPOP1 is overexpressed in 2 of 4 bladder tumors
- We have cloned a large number of F-box proteins using a two-hybrid screen with the Skp1 protein. These will form the basis of an extended project to connect these adapter proteins to their ubiquitylation targets and physiological roles.
- We have published a paper describing the work cloning the variety of F-box proteins (attached). We have submitted a 1 manuscripts on our findings on another F-box protein, called Emi1, that stemmed from this work. We have also completed two manuscripts on related SCF ubiquitin ligases. Other manuscripts are in preparation, but are not yet finished.

REPORTABLE OUTCOMES

- Paper published, attached.
- Manuscripts submitted, Cell, J. Cell Biology, Nature Cell Biology, attached.
- Additional funding was obtained from the NIH for the continued studies on the Emi1 protein
- We are negotiating research collaboration with Genomics Collaborative, Inc. (Cambridge, MA) to further explore the role of F-box proteins in human cancer.

CONCLUSIONS

These studies suggest that hPOP1 may indeed be a tumor suppressor. Additional work including a mouse knock-out and additional expression studies may validate this idea and determine whether hPOP1 may be important in other cancers including prostate cancer. The progress on the project has been modest, in part because we have so few clues about how hPOP1 actually functions. The two-hybrid screen was a first attempt to look for interacting proteins to obtain clues about hPOP1 function. In future, a coimmunoprecipitation/protein sequencing/mass spectrometry approach is more likely to yield interesting information about hPOP1.

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APPENDICES

Regan-Reimann JD; Duong QV; Jackson PK. (1999). Identification of novel F-box proteins in *Xenopus laevis*. *Current Biology* 9, R762-3.

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Correspondence

Identification of novel F-box proteins in *Xenopus laevis*

Julie D. Regan-Reimann,
Quoc Vong Duong and
Peter K. Jackson

The SCF family of ubiquitin ligases [1,2] control many physiological processes including DNA replication [3,4], centrosome duplication [5], kinetochore assembly [6] and transcription [7]. SCFs are composed of four subunits: a protein subunit called a cullin binds two other subunits, one called Skp1 and the other, a ring-finger protein called Rbx1 [8]; Skp1 binds to a protein containing an F box, a 44–50 amino-acid motif found in a variety of proteins [9–12]. To understand the role of SCF complexes in the early embryonic cell cycle, we screened a *Xenopus* oocyte library (Clontech) for F-box proteins using human Skp1 as bait in a yeast two-hybrid screen (the

human and *Xenopus* Skp1 proteins are identical).

In two independent screens (5.3×10^6 clones), 449 positive clones were isolated, from which five novel F-box proteins were identified: Fbx26, Fbx27, Fbx28, Fbl5 and Fbl13. Each had an identifiable F box. A number of other proteins were also identified that interacted with Skp1 but did not fit the current consensus F-box sequence; it is possible that these may turn out to be a distinct class of F-box proteins.

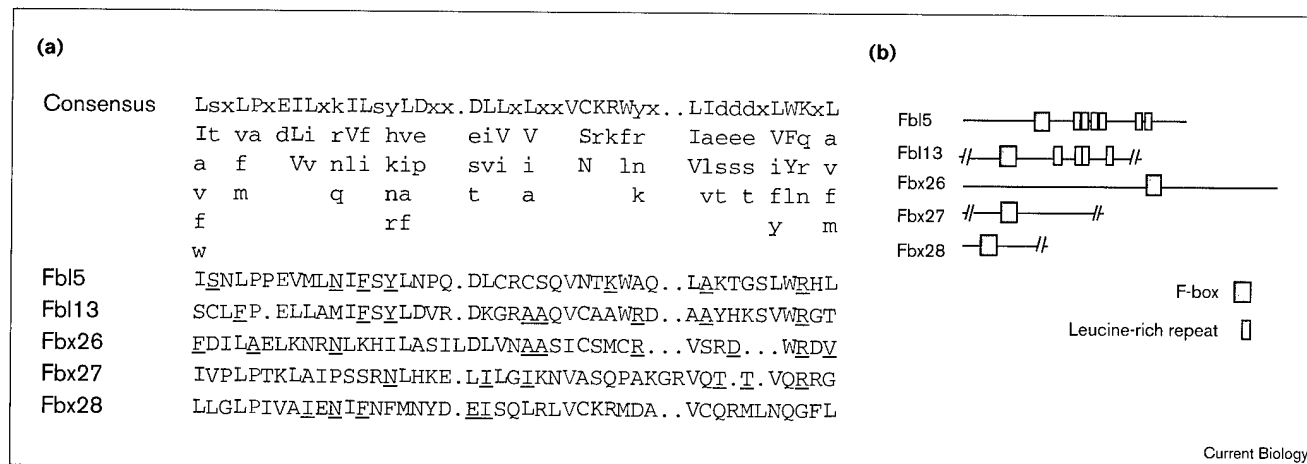
The sequences of the five *Xenopus* F-box proteins are aligned and their domain organizations are shown in Figure 1. Three of the proteins, Fbx26, Fbx27 and Fbx28, did not have recognizable domains outside the F-box motif. Fbx26, which did not bear significant homology to any protein currently in the database, was especially abundant in the library (88% of clones screened). BLAST searches indicated that there was some homology between Fbx27 and the kinetochore protein CENP-F and the retinoblastoma-p105^{Rb}-interacting protein mitotin [13]. Fbx28 showed significant homology

to a human protein, KIAA0483, whose function is unknown.

Two of the proteins, Fbl5 and Fbl13, contained leucine-rich repeats [14]. When the sequence of *Xenopus* Fbl5 was compared with those of the human F-box proteins [9,15], we found that the *Xenopus* protein was most similar to human Fbl5 (61% identity). A phylogenetic comparison (Figure 2) suggested that Fbl5 was related to, but distinct from, Skp2, the other known human Fbl. Skp2 was recently shown to be critical for destroying the cyclin-dependent kinase (Cdk) inhibitor p27^{KIP1} and the transcription factor E2F-1 at the G1–S transition of the cell cycle [7,10–12]. Fbl13 is a novel protein related to, but distinct from, other known F-box proteins in the database that contain leucine-rich repeats (Figure 2).

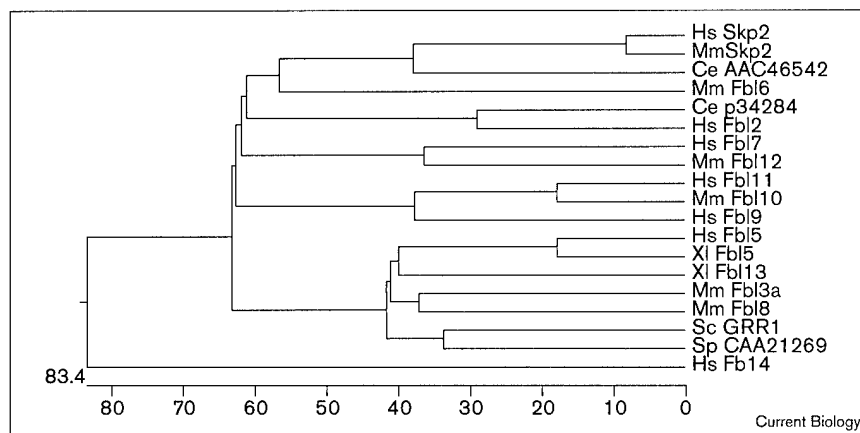
We have identified the first examples of F-box proteins in *X. laevis*. The ability to interfere with the function of proteins in the *Xenopus* embryo by expression of wild-type or dominant-negative versions of the proteins may allow us to identify new roles for SCF ubiquitin ligases in cell-cycle progression and early development.

Figure 1



A family of F-box proteins in *Xenopus laevis*, isolated through their ability to bind Skp1. (a) Alignment of F-box domain sequences. The F-box domains from Fbl5, Fbl13, Fbx26, Fbx27 and Fbx28 were aligned using CLUSTAL V. The consensus sequence is shown above. Red,

most highly conserved; blue, highly conserved; underline, similar. Dots indicate gaps. The clones were isolated the following number of times: Fbl5 (3), Fbl13 (1), Fbx26 (395), Fbx27 (5), Fbx28 (1). (b) Schematic representation of the domain structures of *X. laevis* F-box proteins.

Figure 2

Phylogenetic tree showing evolutionary relationships within the subfamily of F-box proteins characterized by leucine-rich repeats. Ce, *Caenorhabditis elegans*; Hs,

Homo sapiens; Mm, *Mus musculus*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; XI, *X. laevis*.

Acknowledgements

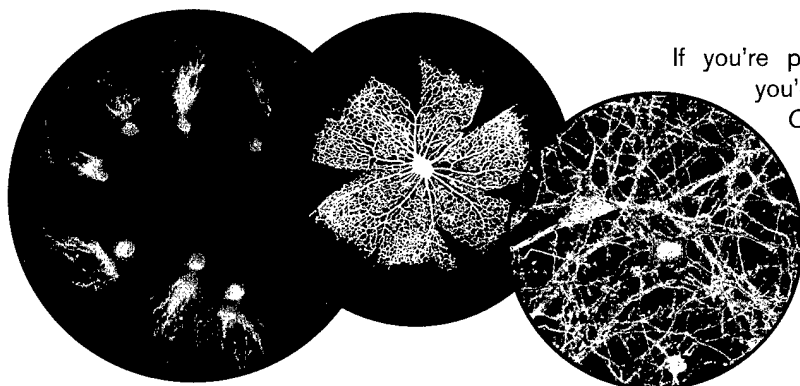
We thank Wade Harper and Michelle Pagano for sharing unpublished results. This work was supported by funding from the NIGMS (GM54811), a junior investigator award from the Howard Hughes Medical Institute (P.K.J.), a grant from the Stanford Cancer Council, and the MSTP training grant (GM07365) from NIGMS (J.D.R.-R.).

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Nuclear accumulation of cyclin E/Cdk2 triggers a concentration-dependent switch for the destruction of p27^{Xic1}

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The action of cyclin-dependent kinases (CDKs) is regulated by phosphorylation, cyclin levels, the abundance of CDK inhibitors, and, as recently has been shown for cyclin B/cdc2, their localization. It is unclear how localization regulates the action of cyclin E/Cdk2 and its inhibitors. Here, we show that the closest known *Xenopus laevis* homolog of mammalian Cdk2 inhibitors p27^{Kip1} and p21^{Cip1}, Xic1, is concentrated, ubiquitinated, and destroyed in the nucleus. Furthermore, Xic1 destruction requires nuclear import, but not nuclear export, and requires the formation of a transport-competent nuclear envelope, but not interactions between the lamina and chromatin. We show that (i) cyclin E/Cdk2 and Xic1 are transported into the nucleus as a complex and that Xic1 destruction requires the activity of cyclin E, (ii) that phosphorylation of Xic1 by cyclin E/Cdk2 bypasses the requirement for nuclear formation, and (iii) that the phosphorylation of Xic1 by cyclin E/Cdk2 is concentration dependent and likely realized through second-order interactions between stable cyclin E/Cdk2/Xic1 ternary complexes. Based on these results we propose a model wherein nuclear accumulation of the cyclin E/Cdk2/Xic1 complex triggers a concentration-dependent switch that promotes the phosphorylation of Xic1 and, consequently, its ubiquitination and destruction, thus allowing subsequent activation of cyclin E/Cdk2.

cyclin-dependent kinase inhibitor | ubiquitin | proteolysis

In vertebrates, the G₁/S transition requires the activity of cyclin E/Cdk2 (1–3). In turn, the abundance of cyclin E, the phosphorylation state of Cdk2, and the abundance of cyclin-dependent kinase (CDK) inhibitors such as p21^{Cip1} and p27^{Kip1} combine to regulate cyclin E/Cdk2 activity. p27^{Kip1} abundance is thought to be largely controlled by its stability (4), suggesting that the destruction of p27^{Kip1} is critical for the G₁/S transition. p27^{Kip1} is a highly conserved protein. The closest known *Xenopus* homolog, p27^{Xic1}, is thought to play a similar role in restraining the G₁/S transition and p28^{Kix1} (a p27^{Xic1} isoform) is up-regulated during gastrulation at the time when the G₁ phase first appears in development (5). The ability of these inhibitors to restrain activation of cyclin E/Cdk2-dependent activation of DNA replication is thus critical for determining the length of G₁.

In *Saccharomyces cerevisiae*, passage through the G₁/S transition is gated by the Cdc28 inhibitor p40^{Sic1}. p40^{Sic1} is destroyed by ubiquitin-dependent proteolysis and targeted for ubiquitination by the Skp1–Cullin–F-box (SCF) ubiquitin ligase complex Cdc34/Cdc53/Skp1/Cdc4 (6–10). The SCF binds to p40^{Sic1} through the F-box protein Cdc4 only after p40^{Sic1} is phosphorylated by a G₁-specific cyclin/Cdc28. Thus, the regulated phosphorylation of the inhibitor controls its stability.

The basic elements of this mechanism in yeast appear to be conserved for regulating p27 stability in vertebrates. In mammalian cells (11, 12), p27^{Kip1} is destroyed by a similar phosphorylation- and ubiquitin-dependent pathway and has been suggested to require Cdc34 (11). However, there may be important differences. In yeast, p40^{Sic1} inhibits an S phase-specific cyclin/CDK complex (Cib5/Cdc28), but is phosphorylated by a

distinct G₁-specific cyclin/CDK complex (Cln2/Cdc28). But in mammalian cells, p27^{Kip1} apparently inhibits and is phosphorylated by the same S phase-promoting complex, cyclin E/Cdk2 (14). Thus, p27^{Kip1} functions as both an inhibitor and a substrate of cyclin E/Cdk2. This dual function creates a conundrum: how can cyclin E/Cdk2, while bound to its inhibitor, phosphorylate the very same molecule, promote its destruction, and thereby be freed to phosphorylate other targets? In other words, is there a molecular switch that changes p27^{Kip1} from an inhibitor to a substrate?

Biochemical studies have begun to address how p27 may act as both inhibitor and substrate. In mammalian cell extracts, p27 is destroyed only when bound to cyclin E/Cdk2 (13). Therefore, the pool of p27 bound to cyclin E/Cdk2 is most critically regulated. At physiological ATP concentrations (≈ 2 mM), p27's substrate activity is favored, whereas lower ATP concentrations favor its inhibitory activity (14). Further, a p27 mutant that binds the cyclin subunit but not the CDK subunit is more readily phosphorylated (15). Thus, cyclin E/Cdk2 may phosphorylate p27 via an intermediate in which p27 is bound to the cyclin but is not yet inhibiting the kinase. However, changes in ATP levels are unlikely to explain the inhibitor-substrate transition inside the cell. Further, the transition rate to the tightly bound state is fast (1 min⁻¹) and the off rate is slow (1/120 min⁻¹) (14), such that the vast majority of cyclin E/Cdk2 and p27 likely exists as an inhibited trimeric complex throughout G₁. Another mechanism must account for the inhibitor-substrate transition.

One such mechanism is suggested by the observation that triggering of DNA replication is tightly coupled to nuclear formation. Moreover, destruction of Xic1 in *Xenopus* egg extract requires addition of sperm chromatin (16). We find that Xic1 also can be both inhibitor and substrate of cyclin E/Cdk2 and that degradation requires association with cyclin E/Cdk2. Is the inhibitor-substrate transition of p27^{Xic1} coupled to nuclear transport and what nuclear-dependent or -independent mechanisms facilitate the transition?

To answer this question, we investigated the effect of nuclear function on Xic1 destruction. We find that Xic1 destruction requires nuclear formation and nuclear transport, that Xic1 and cyclin E accumulate in the nucleus after nuclear formation, and that Xic1 subsequently is ubiquitinated and destroyed in the nucleus, independent of nuclear export. We find that lamina-chromatin interactions required for DNA replication are not required for Xic1 destruction, confirming that Xic1 destruction principally requires nuclear import.

Abbreviations: CDK, cyclin-dependent kinase; GST, glutathione S-transferase; IVT, *in vitro*-translated; MBP, myelin basic protein; MeUb, methylated ubiquitin; LMB, leptomycin B.

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To explain the cyclin E/Cdk2 requirement for Xic1 destruction, we show that cyclin E/Cdk2 phosphorylation of Xic1 bypasses the nuclear requirement for Xic1 destruction, suggesting that the nuclear accumulation stimulates the phosphorylation of Xic1, and that ubiquitination and proteolysis can occur independent of nuclear formation. Finally, because cyclin E/Cdk2 is concentrated in the nucleus before DNA replication (17) we tested and confirmed the model that the effective activity of cyclin E/Cdk2 toward Xic1 depends on the second-order concentration of cyclin E/Cdk2 and Xic1 and likely mediated through interactions between ternary complexes. Based on these results we propose that the facilitated concentration of the cyclin E/Cdk2/Xic1 complex in the nucleus overcomes the inhibitory action of Xic1. This concentration-dependent switch then triggers the phosphorylation and consequent ubiquitination and destruction of Xic1, thereby fully activating cyclin E/Cdk2.

Materials and Methods

Preparation of Interphase Extracts. Interphase extracts were prepared essentially as described (1) but the second spin was performed at 24,000 rpm in a TLS 55 rotor for 15 min at 4°. The golden middle fraction was used. In our hands, these extracts are more reproducibly competent for DNA replication than lower speed extracts.

Destruction and Transport Assays. Destruction assays were conducted as described (16). ³⁵S-labeled Xic1 (0.5 μl/10 μl extract), sperm (3,000/μl), and an energy regenerating system were mixed with extract. Reactions were incubated at room temperature for 2 h and stopped with sample buffer. Samples were resolved by SDS/PAGE, and proteins were transferred to immobilon-P transfer membrane and analyzed by using a Molecular Dynamics PhosphorImaging system.

In transport and destruction assays, reactions were initiated at room temperature and stopped with elution buffer (ELB) (50 mM KCL/10 mM Hepes, pH 7.7/2.5 mM MgCl₂/250 mM sucrose) at indicated times. The diluted extract was immediately overlaid onto 0.5 M sucrose in ELB and spun 20 sec in a horizontal rotor (Beckman 152 centrifuge). The cytoplasmic fraction was removed from above the sucrose cushion and added to sample buffer. The cushion was carefully aspirated. The pellet fraction then was washed once with ELB, spun again, and resuspended in sample buffer. One-fifth of the cytoplasmic and all nuclear samples were resolved by SDS/PAGE, and the proteins were transferred to immobilon-P transfer membrane and analyzed by using a Molecular Dynamics PhosphorImaging system and immunoblotting.

In Vitro Phosphorylation Experiments. Cyclin E/Cdk2 was purified from baculovirus and incubated with Xic1 for 30 min in kinase reaction buffer (100 mM NaCl/20 mM Hepes, pH 7.5/1 mM EDTA/5 mM MgCl₂). Reactions were initiated by the addition of ATP (100 μM) and γ³²P-ATP (1 μM). Reactions were stopped after 3 min with sample buffer. Equivalent volumes were resolved by SDS/PAGE and analyzed by PhosphorImaging.

DNA Replication Assays. Reactions were conducted essentially as described (3) by using trichloroacetic acid precipitation of DNA onto glass fiber filters. Replication efficiency was typically greater than 70%.

Preparation of Recombinant Proteins. Different types of Xic1 proteins [³⁵S-labeled *in vitro*-translated (IVT) Xic1, glutathione S-transferase (GST)-Xic1, and myelin basic protein (MBP)-Xic1] behaved similarly in the assays described. ³⁵S-labeled IVT Xic1 was prepared by using coupled *in vitro* transcription/translation from plasmid pCS2-Xic1. GST-Xic1 and MBP-Xic1 were purified from bacterial strain BL21 pLysS according

to standard protocols. *Xenopus* cyclin E/Cdk2 complex was purified from SF9 cells coinfecting with *Xenopus* cyclin E and *Xenopus* His-Cdk2 expressing viruses (multiplicities of infection of 15 and 10). Cells were harvested in buffer (50 mM Tris-HCl/100 mM KCl/20% glycerol/5 mM MgCl₂/50 mM sodium phosphate/10 mM imidazole, pH 7.7), and the complex was purified on Ni²⁺-nitrilotriacetic acid resin. Peak fractions were pooled and dialyzed into XB (100 mM KCl/10 mM Hepes, pH 7.7) and 20% glycerol. LAP2 fragments were generously provided by Kathy Wilson, Johns Hopkins University School of Medicine, Baltimore (20).

Results

Xic1 Destruction Requires Transport-Competent Nuclei. Sperm chromatin is required for Xic1 destruction (16). To study this requirement in more detail, we tested whether nuclear formation and nuclear transport are required for Xic1 destruction. In these assays, sperm chromatin templates are added to crude egg cytoplasm that includes the vesicular components required for nuclear assembly. The sperm rapidly decondenses (<5 min) and binds vesicles, which then fuse to form the nuclear membrane. Nuclear transport is established by ≈20 min, and DNA replication initiates after ≈30 min. Fig. 1A shows that Xic1 is destroyed in S-phase extracts (lanes 5 and 6) or a reconstituted mix of cytosolic and membrane fractions (lanes 3 and 4), but not in the cytosolic fraction alone (lanes 1 and 2). Thus, Xic1 is destroyed only in extracts with membranes in which the nuclear envelope may form. To test whether Xic1 must enter the nuclear compartment to be destroyed, we added the nuclear transport-blocker wheat germ agglutinin, which blocked Xic1 destruction (not shown), thus confirming our hypothesis. The ability of transport blockers to inhibit Xic1 degradation also supports the idea that the *in vitro*-assembled nuclei specifically import factors for Xic1 destruction and that they are not simply enclosed within assembling nuclei.

Ubiquitination of Xic1 Occurs Within the Nucleus. Although these results suggest that nuclear formation and transport are required for Xic1 destruction they do not show where Xic1 ubiquitination and destruction occur. It is possible, for example, that Xic1 enters the nuclear environment, perhaps to be phosphorylated, but is exported before ubiquitination and destruction. Recent work suggests that overexpressed p27^{Kip1} is destroyed after nuclear export (18). To determine where Xic1 is ubiquitinated and destroyed, we developed a nuclear transport and ubiquitination assay using egg extracts to analyze kinetically how Xic1 and cyclin E/Cdk2 are partitioned between the nuclear and cytoplasmic fractions.

Coupled nuclear assembly-Xic1 destruction reactions containing *Xenopus* egg extract and trace amounts of ³⁵S-labeled Xic1 are initiated by addition of sperm. At various times, nuclear and cytoplasmic fractions are separated by rapid centrifugation, resolved by SDS/PAGE, and analyzed by autoradiography for Xic1 and Western blotting for cyclin E, Cdk2, and other proteins. The kinetics of Xic1 ubiquitination and destruction are best understood in the context of chromatin and nuclear formation in extracts. In the first 15–20 min after sperm addition, chromatin decondenses, and many chromatin-associated proteins including the origin recognition complex (ORC), Cdc6, and the minichromosome maintenance (MCM) proteins, assemble onto chromatin (data not shown); however, Xic1 and cyclin E remain exclusively in the cytoplasm (see Fig. 1B, Xic1_{Cyt}). After ≈20 min nuclear vesicles bind to chromatin and fuse to form a double membrane containing nuclear pore complexes, and nuclear transport is established. By 30 min, Xic1 and cyclin E rapidly accumulate in the nuclear fraction (Fig. 1B, lane 3) and after Xic1_{Nuc} and cyclin E and DNA replication begins (not shown). Shortly after cyclin E and Xic1 begin to accumulate in the

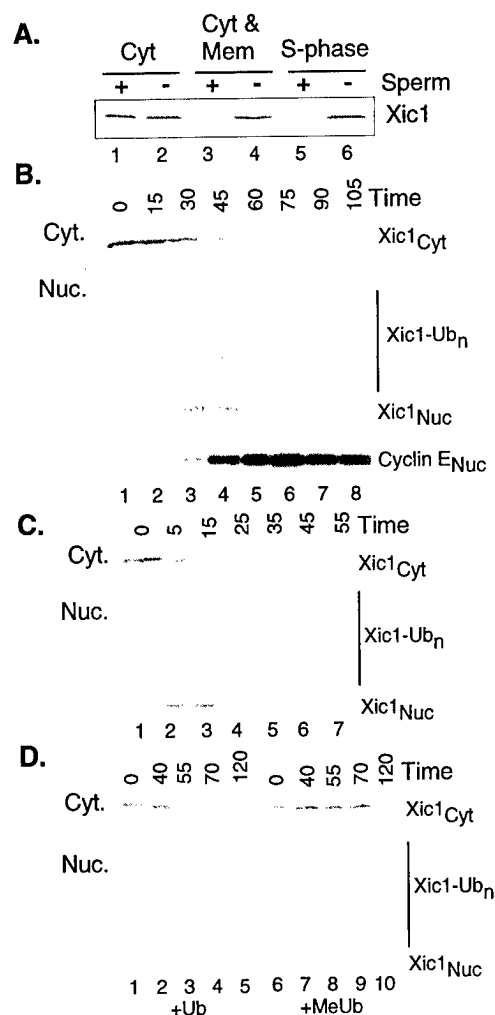


Fig. 1. Xic1 is rapidly transported into and ubiquitinated in the nucleus. (A) Destruction of Xic1 requires formation of nuclei. ³⁵S-labeled IVT Xic1 was added to the indicated extract fraction(s) plus or minus sperm DNA. Reactions were processed and analyzed as described in *Materials and Methods*. Light microscopy confirmed that nuclei formed only in S-phase extract and in the reconstituted cytosolic and membrane fractions (Cyt and Mem). (B) Xic1 is ubiquitinated in the nucleus. Reactions were prepared as in A with sperm DNA, separated into the cytoplasmic and nuclear fractions at the indicated times, and processed as described in *Materials and Methods*. Samples were analyzed by PhosphorImaging or Western blotting with anti-cyclin E antibody. Subtypes of Xic1 are indicated: Xic1_{Cyt} is the cytoplasmic fraction, Xic1-Ub_n is the ubiquitinated nuclear fraction, and Xic1_{Nuc} is the ubiquitinated nuclear fraction. The amount of added IVT Xic1 did not measurably affect the normal time course of DNA replication. (C) Xic1 destruction begins rapidly in preformed nuclei. Sperm and energy were mixed with interphase extract and incubated for 50 min to allow nuclei to form. After nuclear formation was confirmed by microscopy, IVT Xic1 and additional extract were added to the reaction (*t* = 0 min). Samples were removed at indicated times and processed as in B. (D) The modified forms of Xic1 are ubiquitinated. Reactions were prepared as in A and ubiquitin (Ub) or methylated ubiquitin (MeUb) were added and processed at indicated times as in B. To assess the overall effect of MeUb on destruction, comparison of the summed amount of cytoplasmic (Cyt) and nuclear (Nuc) Xic1 remaining was quantitated to be more than 7-fold greater in the sample with added MeUb.

nuclear fraction, slower migrating forms of Xic1 appear (Xic1-Ub_n). These higher forms are rapidly degraded until the overall level of Xic1 is reduced to a background level. The appearance of the slower migrating forms of Xic1 in the nuclear fraction suggests that Xic1 is ubiquitinated in the nucleus.

To confirm that the 30-min time lag before ubiquitinated

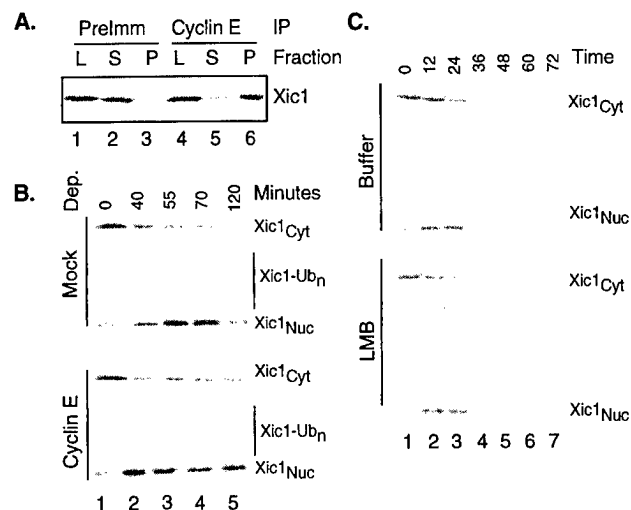


Fig. 2. Xic1 is destroyed inside the nucleus in a cyclin E-dependent manner. (A) Cyclin E and Xic1 form a complex in extract. IVT Xic1 was added to extract. After 30 min the reactions were stopped by dilution into buffer with preimmune or anti-cyclin E sera. After 1 h protein A-Sepharose beads were added. After 20 min the beads were isolated and washed. Load (L), supernatant (S), and pellet (P) fractions were resolved by SDS/PAGE. (B) Immunodepletion of cyclin E blocks ubiquitination and destruction but not nuclear accumulation of Xic1. Mock or anti-cyclin E-depleted (3) extracts were assayed for Xic1 transport and destruction as in Fig. 1. (C) The addition of 1 μM LMB does not alter the kinetics of the nuclear accumulation, ubiquitination, and destruction of Xic1.

forms appear (seen in Fig. 1C) is the result of the process of nuclear formation, we added Xic1 to extracts containing preformed S-phase nuclei (see *Materials and Methods*). Here, the bulk of Xic1 is transported, ubiquitinated, and destroyed in about 15 min (Fig. 1C), confirming that nuclear formation is the rate-limiting step and showing the rapid rate of ubiquitination. In fact, unless we were careful to avoid prematurely mixing radiolabeled Xic1 with the nuclei, ubiquitinated forms appeared almost immediately. Addition of methylated ubiquitin (MeUb), a ubiquitin chain terminator stabilized the upper forms in the nuclear fraction (Fig. 1D). The accumulation of ubiquitinated forms in the nuclear, but not the cytoplasmic fraction, indicates that Xic1 is ubiquitinated in the nucleus.

Association with Cyclin E/Cdk Is Required for Xic1 Destruction. It previously was shown that p21 blocks the destruction of Xic1 (16), suggesting that CDK activity is required. Cyclin E/Cdk2 apparently is required for the destruction of p27^{Kip1} in human tissue culture cells (14). To test whether cyclin E/Cdk2 is required for Xic1 destruction in *Xenopus* extracts, we immunodepleted cyclin E from interphase extracts and performed the Xic1 transport and destruction assay. Fig. 2B shows that in the absence of cyclin E, Xic1 enters the nucleus, but is neither ubiquitinated nor destroyed. Presumably, in these conditions Xic1 is not bound to the cyclin/Cdk complex and, being only 27 kDa, freely diffuses in and out of the nucleus. Here, formation of transport-competent nuclei is the only limiting factor for Xic1 nuclear accumulation and accumulation is only partial. In the control reaction, there is a longer delay before Xic1 reaches its maximal concentration in the nucleus, suggesting that active transport of the cyclin/Cdk2/Xic1 complex after nuclear formation is also a limiting factor. Here, nuclear accumulation of Xic1 is more complete, consistent with active transport. In Cdc34-depleted extracts, Xic1 accumulates in the nucleus at a rate and extent similar to the control depletion, presumably in complex with cyclin E/Cdk2, even though it is not ubiquitinated

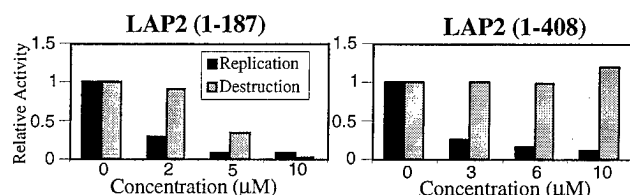


Fig. 3. Disruption of the nuclear lamina does not block Xic1 destruction. Human LAP2 fragments 1–408 and 1–187 (see *Results*) were added to Xic1 destruction and replication assays (see *Materials and Methods*). Destruction activity was defined as the fraction of Xic1 degraded as determined by quantification on the PhosphorImager. Values were normalized to the unperturbed samples.

(data not shown). Thus, it is not merely the nuclear destruction of Xic1 that depletes the cytoplasmic pool, it appears to be the active transport of the cyclin/Cdk/inhibitor complex.

These conclusions require that the majority of Xic1 forms a complex with cyclin E/Cdk2 in extract. To verify this requirement, we immunoprecipitated cyclin E from extract to which exogenous IVT Xic1 was added and examined the soluble and precipitated fractions by SDS/PAGE and autoradiography. Fig. 2A shows that more than 75% of the added Xic1 coprecipitates with cyclin E (lanes 5 and 6), whereas none coprecipitates in a control reaction with preimmune sera (lanes 2 and 3).

Nuclear Export Is Not Required for Xic1 Destruction. It recently was shown in mouse fibroblasts that ectopic expression of the Jab1 protein induces the transport of ectopically expressed p27 from the nucleus into the cytoplasm (18). After nuclear export, p27 is destroyed. In these overexpression experiments, the nuclear export inhibitor leptomycin B (LMB) blocked p27 destruction. The effect of LMB on endogenous p27 destruction with or without Jab1 overexpression was not determined. To test whether nuclear export is required for Xic1 destruction, we added LMB to the transport and destruction assay to a concentration that blocks nuclear export of cyclin B in oocytes (19) and the association of the cyclin B nuclear export sequence with Crm1 in oocyte (19) or egg extracts (data not shown, see *Materials and Methods*). Despite this addition, the kinetics of nuclear accumulation, ubiquitination, and destruction of Xic1 was not delayed (Fig. 2C). Indeed, in many experiments, Xic1 destruction was moderately accelerated in the presence of LMB, suggesting that nuclear export is a back reaction competing with Xic1 destruction, further indicating that Xic1 ubiquitination and destruction occur inside the nucleus.

Disruption of the Nuclear Lamina Blocks DNA Replication but Not Destruction. We considered whether the nuclear lamina also might promote Xic1 destruction. To test this possibility, we perturbed the nuclear assembly-ubiquitination reaction with fragments of human LAP2, an integral nuclear membrane and lamin- and chromatin-binding protein. The addition of 2.5–30 μM of LAP2 fragment 1–187, a region sufficient for chromatin binding, blocks lamin assembly, nuclear import, nuclear membrane fusion, and, thus DNA replication (20). In contrast, addition of 1–3 μM of fragment 1–408, the region sufficient for binding to both chromatin and the lamina, does not block lamin assembly or nuclear import, but inhibits nuclear expansion. Higher concentrations of fragment 1–408 (> 6 μM) blocked DNA replication.

We found that fragment 1–187 blocked DNA replication and Xic1 destruction (Fig. 3 *Left*) in a dose-dependent manner at concentrations similar to those reported. This result was expected because this fragment disrupts nuclear membrane formation. In our hands, fragment 1–408 blocks DNA replication at modest concentrations and, as expected, at high concentra-

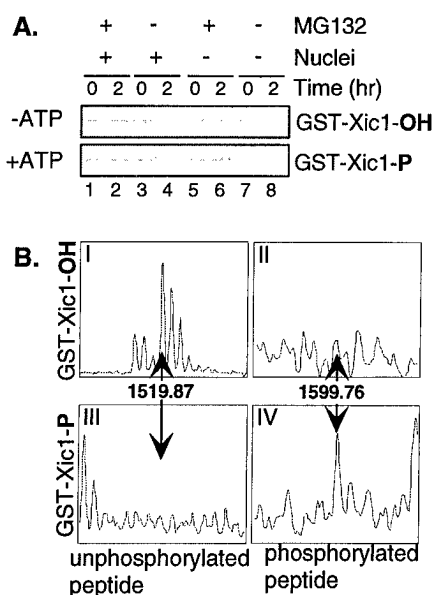


Fig. 4. Prephosphorylation of Xic1 on Thr-205 by cyclin E/Cdk2 bypasses the nuclear requirement for Xic1 destruction. (A) Prephosphorylated Xic1 is destroyed even in the absence of nuclei. GST-Xic1 was incubated with baculovirus-purified cyclin E/Cdk2 in the presence (lower tier) or absence of ATP (upper tier) for 30 min. Destruction assays were conducted plus or minus sperm and MG132 as indicated. Reactions were processed as in Fig. 1A except that membranes were immunoblotted with anti-Xic1 antibody. (B) Cyclin E/Cdk2 phosphorylates Xic1 on Thr-205. Phosphorylated (III and IV) and unphosphorylated (I and II) GST-Xic1 were prepared by kinase reactions with or without ATP (see *Materials and Methods*). Samples were digested with trypsin and analyzed by matrix-assisted laser desorption/ionization–time of flight MS.

tions. However, even at high concentrations Xic1 destruction is unperturbed (Fig. 3 *Right*). This result indicates that whereas both nuclear formation and the function of the nuclear lamina are required for overall DNA replication, destruction of Xic1 requires only formation of the enclosed environment.

Cyclin E/Cdk Phosphorylation of Xic1 on Threonine 205 Bypasses the Nuclear Requirement for Xic1 Destruction. Degradation of Xic1 apparently requires basic steps of phosphorylation, ubiquitination, and proteolysis. We were interested in which of these basic steps depends on nuclear formation and first tested whether the concentrating effect of the nucleus on Xic1 destruction could be mimicked by Xic1 phosphorylation.

To examine this possibility, we prepared prephosphorylated Xic1 protein by incubating a GST-Xic1 fusion protein with cyclin E/Cdk2 in the presence or absence of ATP. After 30 min at room temperature these reactions were added to extract in the presence or absence of the proteasome inhibitor MG132 and the presence or absence of sperm DNA. Fig. 4A shows that if ATP is added and GST-Xic1 is phosphorylated before addition to extract (+ATP: bottom tier), the protein is reproducibly destroyed even in the absence of nuclei (compare lanes 7 and 8). In this experiment, more than 90% of Xic1 is phosphorylated (see below). However, if GST-Xic1 is not prephosphorylated (–ATP: top tier), nuclei are required for its destruction as shown earlier (Fig. 1A). In each case, Xic1 destruction is blocked by MG132, confirming the destruction is proteasome-mediated. Note that although Xic1 binds cyclin E/Cdk2 in the reaction without ATP, it is not destroyed. Therefore, binding is not adequate to bypass the nuclear requirement; phosphorylation is required.

Human p27^{Kip1} requires phosphorylation of threonine 187 for its destruction (14). This phosphorylation site is located within the C-terminal QT domain. Xic1 also has a C-terminal QT

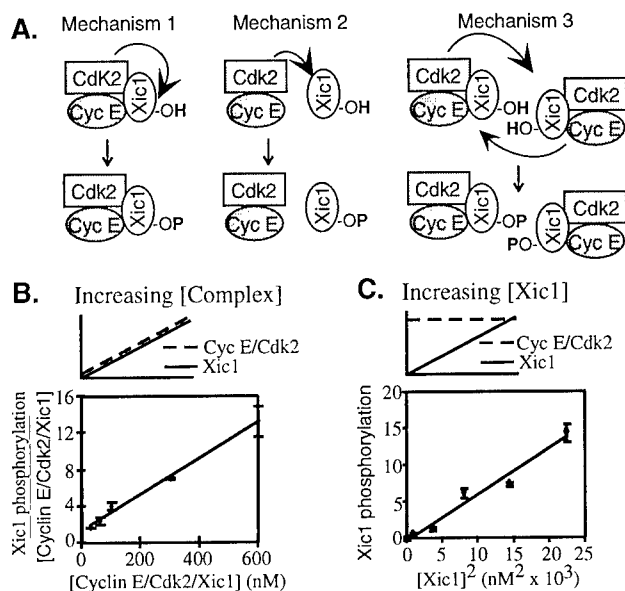


Fig. 5. Phosphorylation of Xic1 by cyclin E is second order with respect to concentration. (A) Schematic models describing mechanisms by which cyclin E/Cdk2 might phosphorylate Xic1. Mechanism 1 is an intracomplex interaction (first order), whereas mechanisms 2 and 3 are intercomplex interactions (second order). (B) Cyclin E/Cdk2 and MBP-Xic1 were mixed in equimolar amounts. After 30 min at room temperature, the reaction was diluted to the indicated concentrations, and kinase reactions were initiated, processed, and analyzed as described in *Materials and Methods*. Values for Xic1 phosphorylation were normalized by concentration and fit to a linear curve ($R^2 = 0.99$). (C) Varying concentrations of MBP-Xic1 were incubated with a fixed concentration of cyclin E/Cdk2 (400 nM). After 30 min the Xic1 phosphorylation reactions were initiated, processed, and analyzed as in A except that the counts were plotted versus the concentration squared through and fit to a linear curve ($R^2 = 0.97$).

domain highly homologous to that of p27^{Kip1}, containing a homologous CDK phosphorylation site, T205 (Fig. 4B) (21), and five other potential CDK phosphorylation sites. To determine the *in vitro* cyclin E/Cdk2 phosphorylation sites, we analyzed tryptic digests of Xic1 phosphorylated *in vitro* by cyclin E/Cdk2 by MS. In the unphosphorylated sample, we observed a strong peak ($\approx 1,519$ Da) corresponding to an unmodified peptide containing threonine 205 (Fig. 4BI), but no peak corresponding to the phosphorylated peptide (Fig. 4BII). In the phosphorylated sample we observed a peak ($\approx 1,599$ Da) corresponding to the phosphorylated peptide (Fig. 4BIV), but not the unphosphorylated peptide (Fig. 4BIII). We did not observe peaks corresponding to phosphorylated forms of any of the other potential Cdk phosphorylated peptides, although we did detect the unphosphorylated peptides containing each of these sites (not shown). These data suggest that cyclin E/Cdk2 phosphorylation of Xic1 on T205 is sufficient to bypass the nuclear requirement for Xic1 destruction. Nonetheless, other mechanisms including phosphorylation-independent mechanisms may be capable of triggering Xic1 degradation.

The Rate of Phosphorylation of Xic1 by Cyclin E/Cdk2 Is Second-Order with Respect to Xic1. We suspected that the active concentration of the cyclin E/Cdk2/Xic1 complex in the nucleus favors Xic1 phosphorylation. To examine this possibility, we investigated the mechanism by which cyclin E/Cdk2 phosphorylates Xic1 *in vitro*.

Cyclin E/Cdk2/Xic1 might phosphorylate Xic1 by at least three mechanisms. First, the cyclin E/Cdk2 complex might directly phosphorylate the inhibitor to which it is tightly bound (cis-phosphorylation) (Fig. 5A Left). Second, free cyclin E/Cdk2 might rapidly phosphorylate free Xic1 (trans-phosphorylation) (Fig. 5A Center). Third, a ternary cyclin E/Cdk2/Xic1 complex

might phosphorylate the inhibitor associated with another similar ternary complex (also trans-phosphorylation) (Fig. 5A Right). A combination of mechanisms is also possible. The first mechanism is independent of cyclin E/Cdk2 and Xic1 concentration, whereas the second and third mechanisms depend on their concentration.

To distinguish the second and third mechanisms from the first, we incubated equimolar amounts of purified cyclin E/Cdk2 and purified MBP-Xic1 in the absence of ATP so that the components could reach their binding equilibrium without undergoing phosphorylation. Because the on-rate is much faster than the off-rate, the great majority of each species is within the ternary complex (data not shown). This reaction was diluted to a range of final concentrations from less than the concentration of endogenous cyclin E in the extract (≈ 60 nM) to many times above this concentration, thereby mimicking the active concentration of the complex in the nucleus. Addition of ATP initiated the phosphorylation of cyclin E and Xic1. Reactions were stopped after 3 min and resolved by SDS/PAGE, and the extent of phosphorylation was quantified. In Fig. 5B, the total phosphorylation of Xic1 is normalized by Xic1 concentration and plotted versus the concentration. This operation yields a linear plot ($R^2 = 0.99$), as would be expected for a second-order reaction, and confirms that increasing concentration increases the intrinsic ability of cyclin E/Cdk2 to phosphorylate Xic1. Therefore, cyclin E/Cdk2 apparently phosphorylates Xic1 by a trans mechanism.

Although this result suggests that Xic1 phosphorylation occurs in trans, it does not determine whether the phosphorylation is mediated through free cyclin E/Cdk2 and Xic1 (mechanism two) or through the ternary complex (mechanism three). To distinguish between these two trans-acting mechanisms, we incubated varying concentrations of Xic1 with a fixed concentration of cyclin E/Cdk2 (400 nM) for 30 min to allow for binding. The phosphorylation reactions then were initiated with ATP. In these reaction conditions, if the phosphorylation occurred through the interaction of the individual components as more Xic1 is added, the phosphorylation of Xic1 would be first-order with respect to the concentration of Xic1. However, if the phosphorylation of Xic1 depends on the formation of the ternary complex cyclin E/Cdk2/Xic1, a second-order interaction is introduced. Consequently, as more Xic1 is added more of the trans-acting ternary complex would form and the phosphorylation of Xic1 would be second-order with respect to the concentration of Xic1. Fig. 5C shows that between 0 and 150 nM plotting the extent of Xic1 phosphorylation versus the square of the concentration of Xic1 yields a linear relationship with R^2 equal to 0.97. If we plot Xic1 phosphorylation versus the concentration (a first-order interaction) R^2 equals 0.87 (consistent with a linear fit to a quadratic). Thus, the data much better fits a second-order dependence on Xic1 concentration. This result suggests that ternary complexes of cyclin E/Cdk2/Xic1 phosphorylate members of other like complexes. Similar results were obtained with purified Xic1 generated by proteolytic cleavage from a GST fusion protein (not shown).

Discussion

We report several observations concerning how the nucleus facilitates the destruction of Xic1, the closest *Xenopus* homolog to p27^{Kip1}. First, we show that Xic1 is ubiquitinated and destroyed inside transport-competent nuclei. In contrast to an earlier study of p27^{Kip1} (18), we show that nuclear export is not required for Xic1 ubiquitination or proteolysis. Second, we show that Xic1 destruction requires cyclin E/Cdk2 activity and that cyclin E/Cdk2 forms a complex with Xic1 in extract. Third, we find that disruption of lamina-chromatin interactions with a fragment of the lamina-associated protein LAP2 does not perturb Xic1 destruction, suggesting that destruction of Xic1 principally requires the formation of a transport-competent nuclear compartment. Fourth, we dem-

onstrate that phosphorylation of Xic1 by cyclin E/Cdk2 bypasses the nuclear requirement for its destruction, suggesting that phosphorylation is the nuclear-dependent step for Xic1 destruction. Last, we find that the phosphorylation of Xic1 by cyclin E/Cdk2 is second-order with respect to the concentration of Xic1.

Based on these results we propose a model by which the active concentration of the cyclin E/Cdk2/Xic1 complex in the nucleus initiates the phosphorylation and destruction of Xic1. This model offers an answer for how nuclear formation contributes to the destruction of Xic1 and, ultimately, the regulation of DNA replication. The model also suggests a means by which CDK inhibitors may function as inhibitors or substrates in distinct cellular compartments.

Nuclear Formation, Xic1 Destruction, and DNA Replication. The observations that nuclear assembly precedes the initiation of DNA replication and that disruption of the nuclear architecture blocks DNA replication as well as many biochemical reconstitution experiments have emphasized the role of the nuclear structure in DNA replication. Ongoing efforts are beginning to elucidate how nuclear formation confers competence for replication.

In this context it was surprising that a soluble extract prepared from crushed nuclei, which is incapable of forming nuclei, is competent to replicate chromosomal DNA (22). This result has been interpreted to indicate that there is no absolute structural requirement for DNA replication. However, it is vital to note that these extracts are prepared from aphidicolin-blocked nuclei that have already fired their origins. Consequently, some of the nuclear requirement for replication before initiation may be bypassed in these extracts.

Our results suggest that in addition to actively concentrating the mechanistic factors responsible for DNA replication, the nucleus concentrates the cyclin E/Cdk2/Xic1 complex to promote the phosphorylation of Xic1. This process may be part of the mechanism by which the nucleus facilitates destruction of Xic1, the subsequent activation of cyclin E/Cdk2, and the initiation of DNA replication. This mechanism would be active before the initiation of DNA replication.

From Inhibitor to Substrate. Our results also address the question of how a CDK inhibitor becomes a CDK substrate at the appropriate moment. The biochemical analysis of Roberts and coworkers (14) demonstrates that p27 can interact with cyclin E/Cdk2 transiently through the cyclin subunit before adopting a tightly bound inhibitory state in which it also binds the CDK subunit. Those authors propose that during this initial interaction, p27 can be phosphorylated. However, they also show that the transition to the inhibitory state is rapid (about 1 min), and the off-rate slow (about 2 h), suggesting that the cyclin E/Cdk2/inhibitor complex exists mostly in the tightly bound state. Therefore, the critical physiological question is how the population of the inhibitor that is tightly bound becomes phosphorylated.

As our work and previous work suggest (14), there is kinase activity associated with the inhibited complex even after equilibrium is reached. Two possibilities explain this observation: first, the more transient CDK interaction alternates between inhibitory and noninhibitory states, thereby allowing for rare phosphorylation of p27; second, a small subpopulation of free kinase may phosphorylate the inhibitor through the transitory interaction described above. Either case is a trans interaction and the rate of trans-phosphorylation will depend on the concentration of both enzyme and substrate. If the complex were only capable of phosphorylating the inhibitor to which it is bound there would be no concentration dependence. Our results confirm that the inhibitor complex is phosphorylated in a concentration-dependent manner. This concentration dependence likely enables cyclin E/Cdk2 to overcome the inhibitory effect of its inhibitor, thereby tipping the balance so that the inhibitor becomes a substrate.

Generating the Switch. Once phosphorylation occurs and the proteolysis pathway is operational a positive feedback loop is established. As the proteolysis machinery destroys the inhibitor a subpopulation of the kinase is activated and able to rapidly phosphorylate more of the inhibitor, leading to more destruction and activation. Therefore, in this scenario, a concentration-dependent switch triggers cyclin E/Cdk2 activation. Advantages to this model are that it evokes only the physiological observation that the cyclin complex is concentrated in the nucleus and the enzymatic details of the cyclin E/Cdk2 p27 interaction.

Multiple Mechanisms for Xic1 Destruction? One prediction of this model is that mutation of critical phosphorylation sites in Xic1 would block Xic1 destruction. In fact, we find that mutation of the six putative serine-proline (SP) or threonine-proline (TP) CDK phosphorylation sites to alanine-proline (AP) does not completely disrupt Xic1 destruction. Nevertheless, as indicated above, cyclin E/Cdk2 phosphorylation of Xic1 bypasses the nuclear requirement. Therefore, there appears to be multiple mechanisms by which Xic1 is destroyed. The mechanism we describe here is phosphorylation-dependent and normally is facilitated by nuclear concentration, but does not strictly depend on the nuclear compartment because this requirement can be bypassed by phosphorylation. The other mechanism is phosphorylation-independent, but apparently occurs normally within the nucleus because we don't observe any cytoplasmic degradation. We currently are working to reconcile these mechanisms, but an interesting possibility is that in the early embryo the phosphorylation dependence is reduced and that one reflection of the appearance of a G₁ phase at the time of gastrulation is an increase in the phosphorylation-dependence for p27 destruction. Indeed, the mechanisms that regulate p27 destruction, including phosphorylation, may be among the most important determinants of the length of G₁.

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Cyclin E Uses Cdc6 as a Chromatin-associated Receptor Required for DNA Replication

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Running Title: ORC/Cdc6 is a chromatin receptor for Cyclin E/Cdk2

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Abstract

Using an *in vitro* chromatin assembly assay in *Xenopus* egg extract, we show that cyclin E binds specifically and saturably to chromatin in three phases. In the first phase, the ORC and Cdc6, but not the MCM, pre-replication proteins are necessary and biochemically sufficient for ATP-dependent binding of cyclin E/Cdk2 to DNA. We find that cyclin E binds the N-terminal region of Cdc6 containing Cy/RXL motifs. Cyclin E proteins with mutated substrate selection/MRAIL motifs fail to bind Cdc6, fail to compete with endogenous cyclin E/Cdk2 for chromatin binding, and fail to rescue replication in cyclin E-depleted extracts. Cdc6 proteins with mutations in the three consensus RXL motifs are quantitatively deficient for cyclin E binding and for rescuing replication in Cdc6-depleted extracts. Thus, the cyclin E-Cdc6 interaction that localizes the Cdk2 complex to chromatin is important for DNA replication. During the second phase, cyclin E/Cdk2 accumulates on chromatin dependent upon polymerase activity. In the third phase, cyclin E is phosphorylated and the cyclin E/Cdk2 complex is displaced from chromatin in mitosis. *In vitro*, MAP kinase and especially cyclin B/Cdc2, but not the Plk1 kinase, remove cyclin E/Cdk2 from chromatin. Rebinding of hyperphosphorylated cyclin E/Cdk2 to interphase chromatin requires dephosphorylation, and the Cdk kinase directed Cdc14 phosphatase is sufficient for this dephosphorylation *in vitro*. These three phases of cyclin E association with chromatin may facilitate the diverse activities of cyclin E/Cdk2 in initiating replication, blocking re-replication, and allowing resetting of origins following mitosis.

Introduction

The requirements for determining the timing and origin selection for eukaryotic DNA replication are now being intensively investigated. In yeast, origin selection requires the origin recognition complex (ORC) to bind initiation sites on DNA (Bell and Stillman, 1992; Rao and Stillman, 1995). Although such initiation sequences are not well defined in higher eukaryotes, it is likely that ORC homologs serve a similar function in these organisms (Carpenter et al., 1996). Studies in *Xenopus* egg extracts and mammalian cells show that ORC recruits Cdc6 and the MCM complex to chromatin (Coleman et al., 1996) and that these preinitiation factors are essential for generating functional origins (Liang et al., 1995; Romanowski et al., 1996; Williams et al., 1997; Yan et al., 1991). The MCM proteins have also been implicated in limiting DNA replication to a single round per cell cycle (Chong et al., 1995; Kubota et al., 1995; Tye, 1994). It is thought that the MCM complex is stripped from chromatin as DNA polymerase moves with the replication fork, thereby removing replication competence from origins that have fired.

The cyclin E/Cdk2 complex is essential for timing initiation of DNA replication (Jackson et al., 1995; Knoblich et al., 1994; Strausfeld et al., 1994) and has been implicated in re-replication control, as high levels of cyclin E appear to block the licensing of origins in *Drosophila* and *Xenopus* (Follette et al., 1998; Hua et al., 1997; Weiss et al., 1998). Concomitant with the initiation of DNA replication, cyclin E is concentrated ~200-fold within the nucleus following nuclear assembly (Chevalier et al., 1996; Hua et al., 1997). The concentration of essential factor(s) such as cyclin E is a central function of the nucleus in DNA replication (Swanson et al., 2000; Walter et al., 1998).

How cyclin E/Cdk2 promotes DNA replication remains unclear, because we do not know its relevant substrates, how those substrates are selected, or how phosphorylation by cyclin E/Cdk2 changes their ability to promote replication. Candidates for cyclin E/Cdk2 substrates have been described, including the protein NPAT (Zhao et al., 1998). Studies from fission yeast show that the Cdc6 homolog, Cdc18, is phosphorylated by a cyclin-dependent kinase at the G1/S transition (Jallepalli et al., 1997) and indeed, human and *Xenopus* Cdc6 are good *in vitro* substrates for Cdk2 kinases (Jiang et al., 1999; Petersen et al., 1999), (Dieter Wolf, LF, and PKJ, unpublished results). Phosphorylation of Cdc6 by a Cdk2 complex in human cells appears to re-localize the Cdc6 protein from the nucleus to the cytoplasm (Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). Although this

relocalization is speculated to inactivate Cdc6 following replication initiation, the specific connection to replication remains unproven.

The ability of cyclin/Cdk complexes to select their specific substrates is determined in part by binding of the cyclin to regions on the substrate. The crystal structure of human cyclin A/Cdk2 bound to the inhibitor/substrate p27^{Kip1} defined a region of the cyclin A protein that interacts directly with p27 (Russo et al., 1996). This region contains the MRAIL motif conserved among cyclin A and cyclin E homologs in many organisms and forms a hydrophobic binding pocket that interacts with an Arg-X-Leu (RXL) peptide within p27. The RXL motif itself is conserved among many cyclin E and cyclin A substrates, including p21, E2F, and p107 (Adams et al., 1996; Chen et al., 1996) suggesting that the RXL motifs are a signature for cyclin/Cdk2 targets. RXL motifs are often surrounded by consensus CDK phosphorylation sites, as is the case for Cdc6 (Jiang et al., 1999; Petersen et al., 1999).

We were interested in further understanding the mechanisms governing cyclin E/Cdk2 control of DNA replication. Because cyclin E/Cdk2 likely phosphorylates chromatin-associated pre-replication proteins, we speculated that cyclin E might function on chromatin. We show here that cyclin E/Cdk2 associates with chromatin in three phases, and that this association in the first phase depends primarily on the prior recruitment of the ORC/Cdc6 complex. We further show that the cyclin E/Cdk2-Cdc6 interaction is a direct association mediated by the MRAIL motif in cyclin E and the RXL motif and possibly another site in the N-terminus of Cdc6, and that this interaction is essential for the initiation of DNA replication. In the second phase, cyclin E/Cdk2 accumulates on chromatin as replication proceeds, potentially explaining the ability of cyclin E/Cdk2 to block re-replication. We find this accumulation requires polymerase activity. In the third phase, the cyclin E-chromatin interaction is abolished in mitosis and reestablished upon the exit from mitosis, thereby allowing a new round of replication. We have found that cyclin B/Cdc2 and to some extent MAP kinase are capable of phosphorylating cyclin E in mitosis and removing it from chromatin, and that Cdc14, a phosphatase essential for the exit from mitosis, is capable of reversing the mitotic phosphorylation of cyclin E and allowing it to rebind chromatin in G1. Thus, the cell-cycle regulated, three-phase association of cyclin E with its chromatin receptor may help explain the coordination of its functions in initiating replication, blocking re-replication, and re-licensing origins.

Materials and Methods

Preparation of Xenopus Egg Extracts and Sperm Nuclei

For interphase extracts, dejellied eggs were rinsed in ELB (250 mM sucrose, 2.5 mM MgCl_2 , 50 mM KCl, 10 mM HEPES pH 7.7, 1 mM DTT, 50 $\mu\text{g/ml}$ cycloheximide, 10 $\mu\text{g/ml}$ cytochalasin D), and centrifuged (13,000 rpm, 10 min). Cytosol was re-centrifuged (24,000 rpm, 10 min) and the supernatant removed with a syringe and kept on ice; the second spin significantly improved replication efficiency. Cycling extracts were made similarly except that eggs were activated by the calcium ionophore A23187 (Sigma) and cycloheximide was omitted from the buffer (Murray and Kirschner, 1989). For chromatin assembly assays, high speed supernatants (HSS) were made similarly, except that XB (50 mM sucrose, 100 mM KCl, 100 μM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, pH 7.7) was substituted for ELB, an energy regenerating system was added, and centrifugation (100,000 x g, 30 min) was performed to remove membranes (Murray et al., 1989). Sperm nuclei were isolated as described (Jackson et al., 1995).

Sedimentation Assays to Isolate Assembled Chromatin from HSS and LSS

HSS reactions: HSS for chromatin assembly was made as described above. Reactions were carried out by incubating 20 μl of interphase HSS with 20 ng of sperm DNA or 1 μg of λ DNA, diluted to 50 μl with XB2 (XB with 2 mM MgCl_2). In some experiments, baculovirus expressed cyclin E/Cdk2 was added to 200 nM. Inhibitors or recombinant proteins were pre-incubated with HSS for 15 min prior to DNA template addition. Upon DNA addition, reactions were incubated (30 min, 22°C), stopped by dilution (150 μl of cold XB2), layered on a 400 μl cushion (1.1 M sucrose in XB2), and spun (11,000 rpm, 30 min, 4 °C) in a SW50.1. The gradient interface was washed with XB2 to remove unpelleted material, and sample buffer was added to the pellet for SDS-PAGE.

LSS reactions: LSS was supplemented with an energy regenerating system prior to sperm addition (1000 sperm/ μl). Samples were incubated (23 °C) for the indicated times, diluted with 5 volumes of cold ELB, layered over a 0.5 M sucrose cushion, and centrifuged in a Beckman 152 microfuge (20 s). Pelleted nuclei were resuspended in sample buffer and analyzed by Western blotting. Chromatin was extracted from a duplicate set of assembled

nuclei by adding 10 volumes of Chromatin Extraction Buffer (50 mM KCl, 50 mM HEPES pH 7.7, 5mM MgCl₂, 5mM EGTA, 2mM β -mercaptoethanol, 0.5 mM spermidine, 0.15mM spermine, 0.1% NP-40), mixing gently, and leaving on ice for 30 min, prior to re-spinning the tubes as above. Protocols provided upon request. Similar assays show the association of replication proteins with chromatin templates (Chong et al., 1995; Martinez-Campa et al., 1997; Yan and Newport, 1995).

Samples treated with mitotic kinases were assembled in LSS (1 h) and murine MAP kinase, human cyclin B/Cdc2 (1 unit each, New England Biolabs), or GST-XPlk-1 (a gift from Jan-Michael Peters, IMP) were added for 10 min. Chromatin fractions were isolated as above.

Replication Assays

10 μ l of cycloheximide-stabilized interphase extract was mixed with 3-5 ng of sperm, and replication assays were performed and quantitated as described (Jackson et al., 1995).

Phosphorylation/Dephosphorylation Reactions

2.5 μ g of bacterially expressed, purified GST-Xcyclin E was incubated with 1 unit of MAP kinase, cyclin B/Cdc2, GST-Plk-1, or baculovirus expressed cyclin E/Cdk2 in Kinase Buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 100 μ M ATP) in the presence of 0.15 μ Ci of [³²P- γ]-ATP. After 30 min at 30 °C, half of each sample was removed and supplemented with 2 μ M GST-Cdc14. Cdc14-treated and untreated samples were incubated (30 min, 30 °C) before stopping reactions with sample buffer, resolving by SDS-PAGE, and visualizing phosphorylated GST-cyclin E by autoradiography.

Calculation of the number of cyclin E molecules per origin

The concentration of cytosolic cyclin E/Cdk2 required for binding to chromatin was estimated by adding baculovirus expressed cyclin E/Cdk2 to DNA in cyclin E-depleted HSS. The number of molecules per origin represented by this binding event was calculated by determining the percentage of cyclin E that bound to DNA by quantitative Western blotting. Assumptions include: the number of origins per nucleus = 10⁵ (Walter and Newport, 1997), the volume of a nucleus = 2.5 n ℓ (Hua et al., 1997), and the

concentration of cyclin E in cytosolic extract = 60 nM and in nuclei = 6 μ M (Hua et al., 1997; Jackson et al., 1995).

Thus, 60 nM/ ℓ cyclin E/Cdk2 \times (6×10^{23} molecules) \times (2.5×10^{-9} ℓ /nuclei)
= (9×10^7 molecules/nuclei)

Because $\sim 0.1\%$ of the cyclin E from HSS binds to chromatin, we estimate:

($\sim 1 \times 10^5$ molecules/nuclei) / (10^5 origins/nucleus) = ~ 1 molecule cyclin E/origin .

To determine the maximum capacity of chromatin for cyclin E, known amounts of baculovirus cyclin E/Cdk2 were titrated into cyclin E-depleted LSS extracts and chromatin-associated cyclin E measured by quantitative Western blot. The maximal level was roughly equal to the amount of endogenous cyclin E bound to chromatin immediately before mitosis.

In vitro Binding Assays

GST fusion proteins of either human (Hu) p21N, Hu p21C, Hu p27, XCdc6N, XCdc6C, or Hu Cdc14, added to a concentration of 1 μ M, were mixed with baculovirus expressed Xcyclin E/XCdk2 (0.4 μ M), and diluted to 10 μ l with XB⁻. Mixtures were incubated (1 h, 25°C), diluted with 90 μ l of IP Buffer (100 mM NaCl, 50 mM β -glycerophosphate, 5 mM EDTA, 0.1% triton X-100, pH 7.2), and spun (13,000 \times g, 10 min). Supernatants were added to glutathione agarose and rocked (30 min, 4 °C). Beads were washed with IP Buffer, resuspended in sample buffer, and resolved by SDS-PAGE.

Mutants of Xcyclin E were created by PCR mutagenesis and verified by sequencing as: M143A L147A W150A and L186A Q187A. RXL mutants of XCdc6 were engineered and verified as 1) R93A L94A L95A, 2) R165A L167A, and 3) R258A L260A.

In vitro translated (IVT) 35 S-labeled cyclin E was expressed from pGEM3Zf+ (Novagen) using the TNT Coupled Reticulocyte Lysate Systems (Promega).

Purification of the XORC Complex from X. laevis Extract

ORC was purified 500-fold from HSS made from the eggs of 50 frogs similar to (Rowles et al., 1996). Modified protocol available upon request.

Immunodepletion and ATP Depletion

Immunodepletions were performed by binding crude (Xcyclin E) or affinity purified (XCdc6) rabbit sera to protein A-Sepharose beads for 1 h. Antibody beads were incubated with extract (2 x 45 min, 4 °C) then centrifuged (13,000 rpm, 10 min). Control depletions were performed with beads alone. ATP depletion was performed by adding hexokinase beads (Sigma) and residual ATP was determined to be <3% by luciferase assay.

Antibody Production and Purification

Purified GST-XORC2, GST-XORC1, GST-Xcyclin E, and GST-XCdc6 were used to raise antisera in rabbits (Josman Immunoresearch, Napa, CA). Affinity purification of antisera was performed by acid elution from MBP-fusion proteins coupled to CnBr-activated sepharose. Anti-Cdk2 antibodies have been previously described (Jackson et al., 1995).

Production of Bacterially Expressed GST- and MBP-proteins and Baculovirus Expressed Cyclin E/Cdk2 and Cdc6.

GST and MBP fusion proteins were expressed in BL21 pLysS and purified over glutathione or amylose resins as described (Jackson et al., 1995).

The following fragments were made as GST- or MBP- fusions: p21N = amino acids 1-90, p21C = amino acids 87-164 (Chen et al., 1995), Cdc6N = amino acids 2-168, Cdc6C = amino acids 169-554 (D. Wolf, unpublished).

Production of baculovirus expressed His-XCdc6 was performed by infecting Sf9 cells with the XCdc6 virus (a gift from Bill Dunphy, Cal Tech), and purifying over Ni-Nta resin (Qiagen).

Baculovirus expressed Xcyclin E/His-XCdk2 (a gift of Jim Maller, U. Colorado) was produced by co-infection with His-XCdk2 virus (MOI=10) and Xcyclin E virus (MOI=15) to favor cyclin E/Cdk2 complex formation (Strausfeld et al., 1996). Autophosphorylated cyclin E was produced by coinfection with Cdk2 at a high MOI for both viruses in the presence of high concentrations of ATP (~1 mM).

Western Blotting

Western blotting was performed as described (Jackson et al., 1995). Affinity purified antibodies were used at 0.5-1.0 µg/ml; crude sera was used as indicated: ORC2 antisera (1:2500), ORC1 antisera (1:2000). Crude MCM3 antisera (1:3000) was a generous gift from Ron Laskey (Romanowski et al., 1996).

Results

Cyclin E/Cdk2 is recruited to chromatin following nuclear accumulation and removed from chromatin in mitosis

To study the ordered events of DNA replication, we optimized an assay to isolate chromatin templates assembled within nuclei formed in low speed supernatants (LSS) of *Xenopus* egg extracts. These "cycling" extracts recapitulate the events of the mitotic cell cycle *in vitro*. First, we separated sperm nuclei assembled in LSS from the cytosolic fraction by centrifugation (Fig. 1A). We extracted purified nuclei with chromatin extraction buffer and re-centrifuged to separate nucleoplasmic proteins from tightly chromatin-associated proteins. Similar assays have been performed in several systems to study the association of replication proteins with chromatin templates (see Materials and Methods). The amount of DNA replication completed at each time point is shown for reference (Fig. 1B). Because cyclin E/Cdk2 promotes DNA replication, we tested whether cyclin E/Cdk2 directly interacts with chromatin. We found that cyclin E/Cdk2 associated with chromatin assembled in cycling LSS extracts (Fig. 1A). In this first phase, cyclin E/Cdk2 was imported into the nucleus following nuclear assembly and bound to chromatin immediately following nuclear import, unlike ORC and Cdc6, which associated with chromatin prior to nuclear formation (Fig. 1A). Cyclin E became detergent-inextractable at the same time that MCMs appear in the detergent-extracted chromatin fractions (not shown).

In a second phase, cyclin E continued to accumulate on chromatin throughout replication (Fig. 1A).

In a third phase, chromatin binding of cyclin E/Cdk2 was mitotically regulated. When cyclin B/Cdc2 kinase activity peaked (indicated by the triangle containing an "M"), cyclin E/Cdk2 was rapidly displaced from chromatin (Fig. 1A). While we saw displacement of XORC1 and XORC2 later in mitosis (not shown), XORC2 appeared to be more stably associated with chromatin in early mitosis (Fig. 1A) when nuclear envelope breakdown was first initiated. Addition of the phosphatase 2A inhibitor okadaic acid (OA) to interphase extracts also induced the mitotic state (Lee et al., 1991) and displaced both cyclin E and XORC from chromatin. Inhibition of cyclin B synthesis and mitotic entry with the protein synthesis inhibitor cycloheximide blocked cyclin E/Cdk2 displacement. Because DNA replication does not require protein synthesis in LSS, this indicates that the

mitotic state, rather than completion of DNA replication, displaces cyclin E/Cdk2 from chromatin. Cyclin E also appears to be more sensitive to mitotic signals for chromatin displacement than XORC.

A chromatin assembly assay shows that cyclin E associates with chromatin with kinetics similar to ORC and Cdc6

To study the first phase of cyclin E/Cdk2 binding to interphase chromatin, we optimized an assay to isolate *Xenopus* sperm or λ DNA templates assembled in high speed supernatants (HSS) of interphase egg extracts (Swedlow and Hirano, 1996). In these extracts, pre-replication complexes (preRCs) form, but events following preRC formation are blocked because the extract lacks membranes and cannot assemble nuclei. We find that *Xenopus* sperm and λ DNA behave identically in all of our HSS assays, which were each repeated using both templates to verify results. The DNA templates used are noted in the figure legends. Following chromatin assembly, reactions were overlaid on a sucrose cushion and chromatin isolated by sedimentation. The chromatin-associated proteins were resolved by SDS-PAGE and examined by Western blotting. The assay was optimized to ensure a high efficiency of isolating the chromatin templates (>95%) and to minimize non-specific sedimentation of cytoskeletal proteins (see Materials and Methods).

In this assay, ORC and Cdc6 associated with chromatin within 5 min, whereas assembly of MCM proteins was consistently delayed, requiring approximately 10 min (Fig. 2). Using sperm or λ DNA, we found the kinetics of assembly were indistinguishable. Single-stranded M13 DNA or RNA was unable to bind pre-initiation factors in this assay.

We found that the endogenous cyclin E/Cdk2 complex bound to chromatin with kinetics similar to ORC and Cdc6 (Fig. 2). On chromatin, cyclin E appeared as a doublet, although the fastest migrating, hypophosphorylated form (see Fig. 9B), bound most readily. Quantitative western blotting indicated that the level of cyclin E/Cdk2 binding to chromatin was ~1 molecule per origin (see Materials and Methods). This low level of cyclin E was difficult to detect and required exposing the blot shown in Fig. 2 overnight. Addition of exogenous cyclin E/Cdk2 purified from baculovirus increased the total amount of cyclin E/Cdk2 bound to chromatin (see Fig. 3B), suggesting that the number of cyclin E/Cdk2 chromatin receptors are in excess in HSS extracts. Nonetheless, addition of excess cyclin E/Cdk2 did not accelerate cyclin E assembly onto chromatin, suggesting that binding

of cyclin E/Cdk2 to chromatin depends on the prior assembly of other factors.

Assembly of cyclin E/Cdk2 onto chromatin requires an ATP-dependent factor in HSS

To determine the requirements for the first phase of cyclin E/Cdk2 binding to chromatin, we incubated a fixed amount of purified baculovirus cyclin E/Cdk2 and λ DNA template with dilutions of HSS, and isolated the assembled chromatin templates. Cyclin E was unable to assemble onto the DNA template in the absence of HSS, but increasing the concentration of HSS caused a linear increase in the amount of cyclin E/Cdk2 assembled onto chromatin (Fig. 3A), suggesting that extract contains an activity that promotes cyclin E binding to chromatin, which we term a "chromatin receptor."

We determined the biochemical requirements for cyclin E/Cdk2 recruitment to DNA (Fig. 3B). Heat treatment or ATP depletion of the extract caused a complete loss of cyclin E/Cdk2 binding to chromatin. ATP depletion (97%) also strongly reduced binding of ORC (Fig. 3B) and Cdc6 (not shown), although a small amount of residual ORC binding to chromatin was observed, likely due to residual ATP-loaded ORC remaining after ATP depletion. Direct binding of yeast ORC to DNA requires ATP (Bell and Stillman, 1992). Addition of excess Mg^{+2} stimulated the assembly of cyclin E/Cdk2 onto chromatin, but not ORC binding (Fig. 3B). Finally, Cdk activity is not required for recruitment, because addition of the chemical Cdk inhibitor roscovitine had no effect on cyclin E chromatin recruitment (not shown). In contrast, protein Cdk inhibitors, including $p21^{Cip1}$ or *Xenopus* $p27^{Xic1}$ (1 μ M) did inhibit cyclin E recruitment to chromatin (not shown) likely indicating that they compete with the endogenous receptor protein(s) for binding to cyclin E (see below).

The ORC/Cdc6 preinitiation complex acts as a receptor for cyclin E/Cdk2 on chromatin

To determine whether preinitiation factors facilitated cyclin E/Cdk2 chromatin recruitment, we depleted ORC, Cdc6, or MCM proteins from HSS prior to the addition of purified cyclin E/Cdk2 and DNA. When the assembled chromatin templates were isolated from these samples, we found that a substantial fraction (about 80%) of the cyclin E/Cdk2 binding was lost in the absence of ORC and Cdc6, whereas MCM depletion had no

significant effect on binding (Fig. 3C). After depletion of ORC or Cdc6, ~ 20% of cyclin E/Cdk2 did bind to chromatin even though ORC and Cdc6 depletions appeared quantitative (>95%, Fig. 3D). We therefore suspect that the ORC/Cdc6 complex may not be the only receptor for cyclin E/Cdk2 on chromatin (see Discussion). Purified *Xenopus* ORC and recombinant XCdc6 rescued cyclin E binding to chromatin from ORC- or Cdc6-depleted extracts (Fig. 3C). Surprisingly, purified ORC, recombinant Cdc6, and an ATP regenerating system incubated with DNA and purified baculovirus Xcyclin E/XCdk2 could reconstitute a large fraction of cyclin E/Cdk2 binding to the DNA template (Fig. 3C). If ORC and Cdc6 were not added, no cyclin E/Cdk2 was recruited to DNA. Thus, in phase one, these two preinitiation factors can function as the cyclin E/Cdk2 receptor on purified DNA.

Recombinant Cdc6 binds directly to the hypophosphorylated forms of cyclin E/Cdk2 in vitro

Recent reports have suggested that human Cdc6 binds efficiently to human cyclin A, but only weakly to cyclin E (Petersen et al., 1999; Saha et al., 1998). However, we find *Xenopus* ORC and Cdc6 are sufficient to bind cyclin E/Cdk2 to DNA. Because ORC recruits Cdc6 (Coleman et al., 1996), we tested whether XCdc6 could bind directly to the *Xenopus* cyclin E/Cdk2 complex. When bacterially expressed GST-XCdc6 was incubated together with baculovirus expressed Xcyclin E/Cdk2, the two proteins efficiently co-precipitated. Addition of an energy regenerating system appeared to stimulate binding but was clearly not essential. Further, the N-terminal half of the Cdc6 protein, which contains all three Cy/RXL motifs (see below) was sufficient for this interaction, whereas the C-terminal portion was not (Fig. 4).

Whereas the specific Cdk inhibitors, p21 and p27, could bind all of the various phosphorylated forms of cyclin E, the N-terminus of Cdc6 preferentially bound the lower (hypophosphorylated) form (Fig. 4), the same form which binds most readily to chromatin. As a control for this type of phosphorylation specificity, we also showed that the cell cycle phosphatase Cdc14, which specifically dephosphorylates mitotically phosphorylated Cdk2 and Cdc2 substrates (BKK, CS, LF, & PKJ, in preparation) binds only the upper, hyperphosphorylated forms of cyclin E, likely because Cdc14 binds to the phosphoserine or phosphothreonine moiety of cyclin E prior to dephosphorylating it.

Thus, the interaction of cyclin E/Cdk2 with Cdc6 appears to be inhibited by cyclin E phosphorylation (see below).

The MRAIL motif of cyclin E is required to bind Cdc6, to facilitate chromatin recruitment, and to initiate DNA replication

RXL/Cy motifs in Cdk substrates and inhibitors are thought to bind to the hydrophobic MRAIL motif in cyclins (Adams et al., 1996; Chen et al., 1996; Russo et al., 1996; Schulman et al., 1998). Comparing Cdc6 protein sequences from *Xenopus*, human, and mouse, we noted the conservation of two "RXL" domains (residues 93-95 and 258-260) in the N-terminal half of the protein, surrounded by consensus Cdk phosphorylation sites, with *Xenopus* containing a third non-conserved RXL motif (residues 165-167). To test whether the interaction between XCdc6 and Xcyclin E is dependent upon an RXL/MRAIL interaction, we first mutagenized the hydrophobic MRAIL domain of the Xcyclin E protein: amino acids M₁₄₃, L₁₄₇, and W₁₅₀, or L₁₈₆ and Q₁₈₇, were mutated to alanine (Fig. 5A). Unlike the wild-type cyclin E protein, neither mutant bound the inhibitor p21 or the substrate Cdc6 *in vitro* (Fig. 5B). Previous studies demonstrated that phosphorylation of RXL-containing cyclin/Cdk substrates require an intact MRAIL sequence in the cyclin, whereas phosphorylation of histone H1 does not (Schulman et al., 1998). We also found that relative to wild type, our cyclin E mutants phosphorylated histone H1 efficiently, but were inefficient at phosphorylating Cdc6 (data not shown). Thus, the mutants retain the activity of properly-folded proteins towards substrates, but substrate selectivity is altered. Further, wild type GST-Xcyclin E could compete with the endogenous cyclin E from HSS for binding to chromatin, but the M₁₄₃ L₁₄₇ W₁₅₀ mutant (Fig. 5C) and the L₁₈₆ Q₁₈₇ mutant (not shown) could not. Therefore, an intact MRAIL domain is necessary to compete for the interaction between cyclin E and chromatin.

Because the MRAIL domain of cyclin E binds Cdc6, we tested whether the MRAIL mutants of cyclin E stimulate replication. We immunodepleted cyclin E from interphase LSS and added back GST fusions of wild-type or MRAIL-mutant Xcyclin E. Whereas the wild-type cyclin protein (30-300 nM) was able to rescue a significant amount of the replication activity in depleted extracts, the mutant protein could not (Fig. 5D). This suggests that the interaction of cyclin E with Cdc6 is essential for DNA replication, although we cannot exclude the possible importance of other substrates of cyclin E/Cdk2

that require the MRAIL motif. Rescue of the cyclin E depletion with the GST-wild-type Xcyclin E protein (45%) was slightly less efficient than rescue with undepleted LSS (59%), which may be due to co-depletion of some of the Cdk2 (See Jackson et al., 1995), although enough Cdk2 remained to combine with the added cyclin E to rescue a substantial fraction of replication activity.

Cdc6 containing mutations in its RXL motifs is quantitatively deficient in binding to cyclin E, phosphorylation by cyclin E/Cdk2, and sustaining DNA replication.

Because the MRAIL motif of cyclin E is required for DNA replication, we tested whether the Cy/RXL region of Cdc6, which likely binds the cyclin E MRAIL motif, was also important for binding to cyclin E and promoting replication. We constructed GST-fusion proteins of XCdc6 containing mutations in one, two, or all three RXL domains, including the first RXL motif: R₉₃, L₉₄, L₉₅, the second: R₁₆₅, L₁₆₇, and the third: R₂₅₈, L₂₆₀ mutated to alanine. The triple RXL mutant of Cdc6, which had the most dramatic phenotype, was quantitatively impaired in its ability to bind to cyclin E (Fig. 6C) and to be phosphorylated by cyclin E/Cdk2 *in vitro* (Fig. 6B), although it retained low levels of both respective activities.

When added to Cdc6-depleted *Xenopus* extracts, the triple RXL mutant failed to efficiently rescue replication at and below the concentration of XCdc6 in extract (Fig. 6A). Adding the triple mutant protein at high levels (>100nM) rescued up to 70% as well as the wild-type protein; however, at and below concentrations at which the wild-type protein sustained significant rescuing activity, the mutant was 1.5- to 5-fold less effective. The lower the concentration of the mutant, the more deficient it was at rescuing replication compared to wild-type Cdc6. The degree to which the mutant was able to rescue replication correlated completely with its level of binding to cyclin E and its level of phosphorylation by cyclin E/Cdk2 *in vitro*. Various combinations of double and single RXL mutants were quantitatively less defective in rescuing replication than the triple mutant, but the degree of rescue consistently correlated with the number of remaining wild-type RXLs (data not shown). The RXL mutants appear to be otherwise functional, as each bound ORC equivalently to wild-type XCdc6 (data not shown).

We also examined a series of Cdc6 N-terminal deletion mutants (see On-line

Supplementary Material). Mutants missing the N-terminal 81 or 108 amino acids of Cdc6 bound cyclin E, were efficient cyclin E/Cdk2 substrates *in vitro*, and stimulated DNA replication. However, mutants lacking 178 or 251 N-terminal amino acids completely failed to bind cyclin E, be phosphorylated, or stimulate DNA replication. These mutants suggested that additional determinants in the amino acid sequence between 108 and 178 (a region which contains only one RXL) are quantitatively important for cyclin E binding and for DNA replication. Each of these truncated Cdc6 proteins bound ORC efficiently, suggesting that they were properly folded to retain other activities. These deletion mutants further support the connection between cyclin E-Cdc6 binding and replication.

We also found that an N-terminal fragment of XCdc6 (amino acids 1-258) containing the cyclin E binding region (Fig. 4) inhibited replication at a concentration of ~300 nM, and completely abrogated replication at ~2 μ M (data not shown). This is comparable to the concentrations of p21 that inhibits replication and ~3.8 times the concentration of endogenous Cdc6 in extract (80 nM, Coleman et al., 1996). Thus, interfering with the cyclin E-Cdc6 interaction, either by mutation of the RXL motifs in Cdc6, by deletions in the N-terminus, or by addition of Cdc6 fragments which bind cyclin E but do not contain the ORC binding region, suppresses replication. Therefore, the first phase of cyclin E recruitment to chromatin by Cdc6 appears to be essential for DNA replication.

Cyclin E accumulation on chromatin depends on polymerase activity.

In a second phase, cyclin E continued to accumulate on chromatin throughout replication (Fig. 1A). Addition of the polymerase α inhibitor, aphidicolin, did not effect the initial binding of cyclin E to chromatin, but blocked the subsequent accumulation step (Fig. 7), indicating that polymerase activity is essential for the accumulation of cyclin E/Cdk2 on chromatin. Addition of aphidicolin had no effect on the level of Cdc6 (Fig. 7) or ORC (not shown) bound to chromatin.

MAP Kinase and cyclin B/Cdc2, but not Plk1, dissociate cyclin E/Cdk2 from chromatin

To further understand the importance of cyclin E/Cdk2 recruitment to chromatin, we wanted to define requirements for the mitotic displacement of cyclin E from chromatin

(third phase). This displacement (Fig. 1A and 7) is consistent with previous data showing that Cdc6 is displaced from mitotic chromatin, and our data showing that Cdc6 is required for cyclin E binding. However, we also noted that hyperphosphorylated cyclin E, as seen in mitotic extracts (see below) does not bind to Cdc6 (Fig. 4).

To determine if any of several essential mitotic kinases were capable of phosphorylating cyclin E and displacing the cyclin E/Cdk2 complex from chromatin, we treated chromatin assembled in interphase LSS extracts with cyclin B/Cdc2, MAP kinase, or the polo-like kinase Plk-1 (Guadagno and Ferrell, 1998; Lane and Nigg, 1996; Murray and Kirschner, 1989) and isolated assembled chromatin. Whereas treatment with Plk-1 had no effect, cyclin B/Cdc2 efficiently removed cyclin E/Cdk2 from chromatin (Fig. 8A). Addition of MAP kinase could also displace the majority of cyclin E/Cdk2 from chromatin, but less efficiently (Fig. 8A). Both cyclin B/Cdc2 and MAP kinase phosphorylated purified GST-cyclin E *in vitro* (Fig. 8B), suggesting that the effect on cyclin E may be direct. Plk1 also phosphorylated GST-cyclin E *in vitro* (Fig. 8B), but the significance of this remains unclear. The Cdc14 phosphatase was capable of reversing the phosphorylation of cyclin E by both Cdc2 and MAP kinase, but not by Plk1 (Fig. 8B), indicating that Plk1 likely phosphorylates cyclin E on different sites than Cdc2 and MAP kinase.

The mitotic phosphorylation of cyclin E that blocks chromatin recruitment can be reversed by the Cdc14 phosphatase

We had previously found that during mitosis cyclin E/Cdk2 is hyperphosphorylated on the cyclin and is ~3-fold increased in activity. This mitotic hyperphosphorylation is inhibited by the Cdk inhibitor p21, indicating that this phosphorylation is Cdk-dependent, likely by one of the mitotic Cdk activities in eggs: cyclin A/Cdc2, cyclin B/Cdc2, or cyclin E/Cdk2 (A Sherman and PKJ, unpublished data). Cyclin E/Cdk2 can also autophosphorylate on the cyclin. To correlate the changes in the phosphorylation of cyclin E with mitotic events, we examined the mobility of cyclin E from mitotic or interphase extracts by SDS-PAGE, visualized by Western blotting. Cyclin E was present in at least two forms in interphase extract. Addition of the phosphatase 2A inhibitor and mitotic inducer, okadaic acid, (Goris et al., 1989), resulted in hyperphosphorylation of cyclin E, as did addition of a non-destructible form of cyclin B. This phosphorylation was reversed

by the mitotic phosphatase, Cdc14 (Fig. 9A). Cdc14 has been found to be important for the exit from mitosis and appears to function by dephosphorylating substrates of cyclins E, A, and B (BKK, CS, LF and PKJ, manuscript in preparation). *in vitro*, Cdc14 can directly dephosphorylate cyclin E that has been previously phosphorylated by MAP kinase, cyclin B/Cdc2, or cyclin E/Cdk2 autophosphorylation, but not Plk1 (Fig. 8B).

To test whether phosphorylation of cyclin E affected chromatin binding, we prepared uniformly autophosphorylated cyclin E/Cdk2 (see Materials and Methods). We observed that hyperphosphorylated cyclin E/Cdk2 was unable to bind to chromatin, even in the presence of HSS (Fig. 9B). Because Cdc14 can reverse the mitotic phosphorylation of cyclin E *in vitro* (Fig. 8B) and because Cdc14 is required for mitotic exit in yeast (Visintin et al., 1998; Wood and Hartwell, 1982), we tested whether Cdc14 would also promote the binding of hyperphosphorylated cyclin E to chromatin. We treated hyperphosphorylated cyclin E/Cdk2 with the Cdc14 phosphatase or with calf intestinal phosphatase (CIP) as a control. Only Cdc14, and not CIP, was able to dephosphorylate cyclin E (Fig. 9B). The collapse of bands seen in Figure 9B upon treatment of cyclin E with Cdc14 corresponds to dephosphorylation of cyclin E. When the phosphatase-treated fractions of cyclin E/Cdk2 were tested in the chromatin assembly assay, only the Cdc14-treated, dephosphorylated cyclin E bound to chromatin, whereas untreated and CIP-treated fractions did not (Fig. 9B). Thus, Cdc14 or a similar phosphatase may dephosphorylate mitotic cyclin E/Cdk2 to allow chromatin binding after mitosis, setting up a new round of DNA replication.

Discussion

Cyclin E/Cdk2 binds to a saturable chromatin receptor composed of ORC, Cdc6, and possibly other factor(s)

We have detailed the requirements and cell cycle behavior of the cyclin E/Cdk2-chromatin interaction. A previous study did not see cyclin E associating with chromatin (Hua et al., 1997). This study showed that in buffers containing the detergent Triton X-100 and lacking chromatin stabilizing factors such as spermine, spermidine, and ATP, the ORC complex remained bound to chromatin but that no cyclin E was observed. Under these specific conditions, we find that cyclin E/Cdk2, and both Cdc6 and MCM3, are stripped from chromatin (LF and PKJ, unpublished results). However, our data is consistent with a previous immunofluorescence study, which observed that cyclin E co-localizes with de-condensed, but not mitotic chromatin (Chevalier et al., 1996).

There are several reasons why we observe modest levels of cyclin E binding to chromatin in the absence of the nucleus and why we need to add exogenous cyclin E/Cdk2 to see a strong signal in our HSS chromatin binding assay. Although the major constituents of the cyclin E chromatin receptor, ORC and Cdc6, bind to chromatin with high affinity in membrane-free extracts, we find that nuclear import, or a step subsequent to it, is required for cyclin E/Cdk2 to bind chromatin efficiently. Newport and colleagues have shown that cyclin E is concentrated 200-fold in the nucleus (to $>5\ \mu\text{M}$) upon nuclear assembly (Hua et al., 1997). The cyclin E-Cdc6 interaction appears to be of sufficiently low affinity to require the active concentration of cyclin E to drive its chromatin association. We find that cyclin E binds to Cdc6 with much lower apparent affinity than to p21 (Fig. 4 and 5B). Additionally, because cyclin E directs ubiquitylation and destruction of its bound inhibitor p27^{Xic1} only on chromatin (LF, CS, BKK, and PKJ, manuscript submitted) following nuclear accumulation of the cyclin E/Cdk2/Xic1 complex (Swanson et al., 2000), an SCF activity important for p27^{Xic1} destruction may need to be associated with cyclin E/Cdk2 to help recruit or stabilize the cyclin E complex on chromatin. SCF activity towards p27^{Xic1} requires the prior assembly of ORC, Cdc6, and MCM proteins onto chromatin (LF, CS, BKK, and PKJ, manuscript submitted). Possibly, Cdc7 is also required, resulting in a sequential link whereby Cdc7 acts prior to Cdk2 activation to trigger replication initiation, as was recently observed (Jares and Blow, 2000; Walter, 2000).

ORC/Cdc6 thus is one, but may not be the only, receptor for cyclin E/Cdk2 on chromatin. Our reconstituted chromatin binding reaction allowed us to show that ORC and Cdc6 are required for the first phase of cyclin E/Cdk2 recruitment to chromatin. Residual cyclin E/Cdk2 binding to chromatin in the absence of ORC or Cdc6 suggests that there may be other yet unidentified factor(s) that recruit cyclin E/Cdk2 to chromatin.

By quantitating the amount of cyclin E bound to chromatin during the cell cycle, we gain possible insight into cyclin E's multiple roles in promoting initiation, preventing re-replication, and allowing origin resetting. During the first phase, we see binding of cyclin E/Cdk2 to chromatin at ~100 nM, just above the concentration of cyclin E in interphase cytosol (~60nM, Hua et al., 1997). This explains why we see only minimal binding of cyclin E to DNA in HSS, and why cyclin E/Cdk2 must be concentrated in the nucleus to facilitate full binding. The chromatin receptor for cyclin E appears saturated when exogenous cyclin E/Cdk2 is added to HSS at ~1 μ M, approximately the concentration of cyclin E/Cdk2 found in the nucleus soon after nuclear formation. This level of cyclin E/Cdk2 binding to chromatin corresponds to about 1 cyclin E molecule per origin during early replication. As replication proceeds, cyclin E/Cdk2 is deposited on chromatin, dependent on the action of polymerase. We find ~5-10-fold more cyclin E binds by the end of replication (see Materials and Methods for calculations.) This wide range of cyclin E/Cdk2 binding, beginning with low binding in phase one before origins have fired and increasing to high levels throughout phase two as replication proceeds, provides a potential mechanism for the observations that cyclin E both promotes initiation and prevents re-replication. The chromatin substrates of cyclin E/Cdk2 that become phosphorylated to initiate replication or to block re-replication remain unknown, but ORC and Cdc6 themselves are reasonable candidates (see below).

Cyclin E uses its MRAIL motif to bind Cdc6 N-terminal/RXL sequences, an interaction important for DNA replication

Our data suggest that the interaction between cyclin E and Cdc6 on chromatin is essential for DNA replication. Work in yeast has also shown that the N-terminal 47 amino acids of Cdc6 interact with the Cdk complex that promotes initiation in *S. cerevisiae*, Clb5/Cdc28. However, the Cdc6/Cdc28 interaction in *S. cerevisiae* appears to be a complicated one, required at physiological levels of Cdc6 but not when the Cdc6 protein,

missing the N-terminal 47 amino acid minimal binding domain for Cdc28, is overexpressed (Elsasser et al., 1996). This work compliments our study, suggesting that the strength of the Cdc6/Cdk interaction is concentration-dependent and likely indicating that a domain beyond the N-terminus of *S. cerevisiae* Cdc6p is also involved in binding Cdc6 to Cdk complexes, but with lower affinity. Although a canonical Cy/RXL motif of *S. cerevisiae* Cdc6 lies very close to the N-terminus, a second RXL motif can be found in the middle of the protein.

N-terminal deletions of Cdc6 and mutations in the RXL motif cause strong or moderate loss of cyclin E/Cdk2 binding and a parallel loss in the ability of these Cdc6 variants to stimulate DNA replication. There may be important determinants for cyclin E-Cdc6 interact in residues 108-178, independent of the Cdc6 RXL motifs.

Our work also suggests a correlation between phosphorylation of Cdc6 and DNA replication. In yeast, phosphorylation of Cdc6 has been shown to play a role in its destruction (Elsasser et al., 1999). In human cells, cyclin E phosphorylates Cdc6 *in vitro* and *in vivo* at three sites in the Cdc6 N-terminus, close to the RXL motif, and phosphorylation by Cdks appears to control the localization of Cdc6 (Jiang et al., 1999; Saha et al., 1998). In studying the various combinations of RXL mutants in *Xenopus* Cdc6, we noticed a strong correlation between the degree of *in vitro* phosphorylation of the XCdc6 mutants by cyclin E/Cdk2 and the amount of DNA replication sustained by each mutant in Cdc6-depleted extract. A recent report found that an unphosphorylatable mutant of XCdc6 supports a single round of DNA replication (Pelizon et al., 2000). Nonetheless, the quintuple serine mutant used in the study by Pelizon et al. still contains intact threonine residues that are part of Cdk consensus sequences, and may therefore sustain a low but sufficient level of phosphorylation to promote replication. However, mutation of the five serine residues does prevent nuclear export of Cdc6. Thus, phosphorylation of Cdc6 by cyclin E/Cdk2 (or in human cells, cyclin A/Cdk2) may occur after initiation, causing Cdc6 to exit the nucleus to prevent re-replication. This is consistent with our model, wherein a build up of cyclin E/Cdk2 on chromatin, coincident with the movement of polymerase, could allow concentration-dependent phosphorylation of Cdc6 on chromatin to dislodge or promote destruction of the Cdc6 protein. Our results show that Cdc6 must recruit cyclin E/Cdk2 to chromatin for efficient replication. The results of Pelizon et al argue that Cdc6 phosphorylation by cyclin E/Cdk2 is not positively required for replication. Together,

these data suggest that the interaction between cyclin E/Cdk2 and Cdc6 may be biochemically distinct from a kinase-substrate interaction; instead Cdc6 may serve to recruit or organize cyclin E/Cdk2's ability to direct downstream events of origin unwinding.

In human USO2 cells, cyclin A, rather than cyclin E, mediates the majority of Cdc6 phosphorylation by Cdks (Petersen et al., 1999). This may simply reflect differences between human somatic cells and amphibian eggs. Note that in human cells, cyclin A is a primary partner of Cdk2, whereas in *Xenopus* eggs, ~90% of the Cdk2 is associated with cyclin E (Jackson et al., 1995) and cyclin A is complexed with Cdc2 (Minshull et al., 1989).

Mitotic regulation of the cyclin E/Cdk2 chromatin association may be an important mechanism in re-replication control

We found that mitotic cyclin E hyperphosphorylation apparently causes the cyclin E/Cdk2 complex to be removed from chromatin. Several arguments suggest that cyclin B/Cdc2 directly phosphorylates cyclin E in mitosis to cause its displacement from chromatin. First, cyclin E disappears from chromatin after replication is complete (Fig. 1A and 7) when high levels of cyclin B/Cdc2 activity indicate that the extracts are in mitosis. Second, cyclin E is unable to associate with chromatin assembled in CSF-arrested mitotic extracts in the absence of calcium (LF & PKJ, unpublished) when cyclin B kinase activity is high. Third, the dissociation of cyclin E/Cdk2 from chromatin assembled in cycling extracts can be blocked by cycloheximide addition, which prevents cyclin B synthesis and entry into mitosis (Fig. 1A). Finally, addition of cyclin B/Cdc2 to fully-assembled interphase chromatin removes cyclin E from the chromatin template (Fig. 8A). The ability of cyclin B/Cdc2 to phosphorylate recombinant cyclin E *in vitro* (Fig. 8B) suggests that this effect is direct, rather than an indirect result of inducing mitosis. MAP kinase addition can also dissociate cyclin E from chromatin, although less efficiently than cyclin B/Cdc2 (Fig. 8A). This result may indicate that MAP kinase is important for keeping cyclin E from rebinding to chromatin in late mitosis, when MAP kinase functions to maintain the mitotic state following Cdc2 inactivation (Guadagno and Ferrell, 1998). It has been observed that the activity of cyclin E/Cdk2 is ~3 fold higher in mitosis (Fang and Newport, 1991; Jackson et al., 1995). Thus, it is possible that cyclin E/Cdk2 autophosphorylation contributes to its mitotic displacement. The essential mitotic kinase Plk1, a homolog of

Drosophila polo, does not appear to affect cyclin E chromatin binding.

Dephosphorylation of cyclin E by Cdc14 reverses the effects of the mitotic kinases and promotes cyclin E/Cdk2 binding to chromatin. In budding yeast, Cdc14 plays an essential role in the exit from mitosis (Visintin et al., 1998) in part by reversing the mitotic phosphorylation of Cdk substrates. We have found that Cdc14 plays a similar role in vertebrates (BKK and PKJ, unpublished). Thus, the dephosphorylation of cyclin E by Cdc14 following mitosis may provide one explanation for how Cdc14 promotes mitotic exit. However, Cdc14 may not be the only phosphatase capable of increasing the amount of cyclin E on chromatin. Phosphatase 1 (PP1) is also capable of dephosphorylating *Xenopus* cyclin E *in vitro* (Rempel et al., 1995) and is also important for progression out of mitosis (Maller, 1994).

The regulation of cyclin E/Cdk2 chromatin association by phosphorylation may help explain how cyclin E mediates re-replication control. Oscillations in the level of cyclin E/Cdk2 are required for *Drosophila* endocycles, as constitutive expression of cyclin E in *Drosophila* salivary glands inhibits cell growth and further rounds of DNA replication (Follette et al., 1998). A similar phenomenon was reported in *Xenopus* extracts, which are unable to replicate in the presence of high levels of cyclin E/Cdk2 (Hua et al., 1997). We show that in phase two, cyclin E accumulates on chromatin as replication progresses (Fig. 1A) and that chromatin accumulation of cyclin E can be blocked at stage one levels by addition of the polymerase α elongation inhibitor, aphidicolin (Fig. 7). Our data is thus consistent with cyclin E/Cdk2 playing a role in both initiation and re-replication control, since it appears to bind additional chromatin receptor(s) as replication progresses, and to be stripped from chromatin via phosphorylation by Cdc2 and/or MAP kinase in mitosis. In the next cell cycle, a permissive state for cyclin E/Cdk2-chromatin binding may be re-established by Cdc14 dephosphorylation of cyclin E upon the exit from mitosis and entry into G1 (for model, see Fig. 10).

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Legends

Figure 1. Cyclin E associates with chromatin in LSS following nuclear import.

A. Sperm chromatin was assembled in the presence of cycling LSS at 23°C from 0 to 2 hours (time of assembly shown beneath blots), before spinning through a sucrose cushion to isolate nuclei in duplicate. One nuclear sample was extracted with chromatin extraction buffer and re-spun to isolate chromatin-associated proteins. Cytosolic, nuclear and chromatin-associated samples were resolved by SDS-PAGE and analyzed by Western blotting with ORC or cyclin E antibodies. Schematics above blots depict the timing of relevant events including nuclear import (NI), DNA replication, cyclin E association with chromatin, and mitosis (M). The indicated samples were supplemented with 10 μ M okadaic acid (OA) or 100 μ g/ml cycloheximide (CHX) for 120 min.

B. Samples identical to those in Part A were supplemented with [α - 32 P]-dCTP. At each timepoint, the reactions were stopped and the amount of DNA synthesized in duplicate samples was quantitated as detailed in Materials and Methods.

Figure 2. The cyclin E/Cdk2 complex from HSS associates with chromatin with kinetics similar to ORC and Cdc6, but earlier than MCM3.

Chromatin was assembled by addition of sperm DNA to high-speed supernatants (HSS) from *Xenopus* egg extracts and reactions were stopped at indicated times. Chromatin templates were isolated, resolved by SDS-PAGE, and analyzed by Western blotting with antibodies to *Xenopus* ORC2, Cdc6, MCM3, and cyclin E (see Materials and Methods). Lane 1: No DNA, 30 minutes. Lanes 2-5: DNA templates assembled for 0, 5, 10, or 15 min. Later time points showed no additional assembly of ORC, Cdc6, MCM3, or cyclin E/Cdk2.

Figure 3. To assemble onto chromatin, cyclin E/Cdk2 requires an activity present in HSS that minimally contains ORC and Cdc6.

A. HSS was diluted with XB2 buffer prior to the addition of λ DNA templates and baculovirus cyclin E/Cdk2 for a 30 min incubation. Assembled chromatin was isolated and analyzed as in Fig. 1. Lane 1: No DNA. Lane 2-6: DNA templates assembled in HSS that was undiluted, or diluted 1:1, 1:3, 1:7, or 1:11 with XB2.

B. HSS was either left untreated (lanes 1-3), heat-treated (lane 4), ATP-depleted (lane 5), or supplemented with 10 mM $MgCl_2$ (lane 6) prior to the addition of λ DNA templates (lanes 2-6). Purified baculovirus expressed cyclin E/Cdk2 was also added to samples in lanes 3-6. Assembled chromatin was isolated and analyzed as above.

C. Individual aliquots of HSS were immunodepleted with antibodies specific to XORC2 (lane 3, 6), XCdc6 (lane 4, 7), XMCM3 (lane 5), or with beads alone (lane 2). Specific samples were supplemented with purified XORC complex (lane 6, 8) or baculovirus expressed XCdc6 (lane 7, 8). All samples included baculovirus expressed Xcyclin E/Cdk2 and an energy regenerating system. Depleted samples with and without additions were incubated with λ DNA for 30 min, sedimented through a sucrose cushion, and resolved by SDS-PAGE.

D. Western blots of depleted HSS used for assembling chromatin in part C. Lane 1: mock depleted; lane 2: ORC2 depleted; lane 3: Cdc6 depleted; lane 4: MCM3 depleted.

Figure 4. Purified cyclin E/Cdk2 binds directly to Cdc6.

Baculovirus expressed cyclin E/Cdk2 was incubated for 30 min with an energy regeneration system and purified GST fusion proteins including: GST-p21N₁₋₉₀ (lane 1), GST-p21C₈₇₋₁₆₄ (lane 2), GST-p27 (lane 3), GST-Cdc6N₂₋₁₆₈ (lane 4), GST-Cdc6C₁₆₉₋₅₅₄ (lane 5), GST-XORC1 (lane 6) or GST-hCdc14 (lane 7). Reactions were diluted in IP Buffer and bound to glutathione agarose beads. Beads were washed, resolved by SDS-

PAGE, and cyclin E was visualized by Western blotting.

Figure 5. The MRAIL motif of cyclin E is required for binding of cyclin E to Cdc6, recruitment of cyclin E/Cdk2 to DNA, and replication competence.

A. Schematic of the *Xenopus* cyclin E protein. The shaded area indicates the cyclin box, and within this region, mutations made for these experiments are demarcated with *'s for the MLW mutant and ^'s for the LQ mutant, and amino acid numbers are listed above. Putative phosphorylation sites are also depicted, preceded by the amino acid number of the specific serine or threonine residue.

B. Wild-type *Xenopus* cyclin E (lanes 1-2 and 5-6), or cyclin E with mutations in the MLW (lane 3, 7) or the LQ (lane 4, 8) peptide sequences were radiolabeled by *in vitro* translation (IVT) in rabbit reticulocyte lysate. The IVT cyclin E variants were added to bacterially expressed GST-p21N (lanes 1-4) or GST-XCdc6 (lanes 5-8), incubated for 30 min, and diluted in IP buffer. GST-p21- and GST-Cdc6-associated cyclin E/Cdk2 was precipitated with glutathione agarose beads. Beads were washed, resuspended in sample buffer, proteins were resolved by SDS-PAGE, and visualized by autoradiography. Lanes 9-11 show a matched exposure of the amount of input IVT cyclin E used in the binding experiments.

C. HSS was supplemented with buffer (lanes 1-2), with 100 nM GST (lane 3), or with increasing doses (30, 60, or 100 nM) of wild-type (lanes 4-6), or MRAIL-mutant (lane 7-9) GST-Xcyclin E. After pre-incubating the HSS with the GST proteins, λ DNA templates were added to extracts (lanes 2-9), and assembled chromatin was isolated and resolved by SDS-PAGE, and blotted for the presence of endogenous cyclin E. The lack of a cyclin E signal in lanes 5 and 6 indicates that wild-type GST-Xcyclin E can effectively compete away chromatin binding of endogenous cyclin E at the indicated concentrations.

D. LSS was immunodepleted with cyclin E antibodies conjugated to Protein A-Sepharose beads. Depleted samples were supplemented with undepleted LSS or increasing

concentrations of wild-type or MRAIL-mutant GST-Xcyclin E as noted, prior to the addition of sperm DNA, an energy regenerating system, and [α - 32 P]-dCTP. Replication was assayed and quantitated in duplicate samples as described in Materials and Methods and plotted as a percent, normalizing the amount of replication in undepleted LSS to "100%." This corresponds to 1.7 ng/ μ l of new DNA synthesized from the 2.5 ng/ μ l of DNA added.

Figure 6. RXL mutants of Cdc6 show a quantitative defect in their ability to bind to cyclin E, to get phosphorylated by cyclin E/Cdk2, and to sustain replication in Cdc6-depleted extract.

A. LSS was immunodepleted with affinity purified XCdc6 antibodies conjugated to Protein A Sepharose beads. Depleted samples were supplemented with sperm DNA, an energy regenerating system, [α - 32 P]-dCTP, and 1, 5, 10, 20, 30, or 100 nM of either wild-type GST-XCdc6 (diamonds) or GST-XCdc6 with all 3 RXL motifs mutated to AXA (squares; see Materials and Methods for mutant description). Replication was quantitated as indicated in Materials and Methods and plotted as a percent of undepleted extract, normalizing to 100% rescue in mock-depleted extracts and setting 0% replication as the amount of background counts incorporated after depletion.

B. Purified GST (lane 1), wild-type GST-XCdc6 (lane 2), or triple RXL-mutant GST-XCdc6 (lane 3) was incubated with purified baculovirus expressed cyclin E/Cdk2 in the presence of [32 P- γ]-dATP. Proteins were resolved by SDS-PAGE and phosphorylated proteins visualized by autoradiography. Membrane stained with Ponceau S is shown below as a loading control.

C. Purified GST (lane 1), wild-type GST-XCdc6 (lane 2), or triple RXL-mutant GST-XCdc6 (lane 3) was incubated with radiolabeled IVT Xcyclin E. After a 30-min incubation, samples were diluted in IP buffer, GST-proteins were precipitated with glutathione agarose beads and washed. Beads were resuspended in sample buffer and associated proteins were resolved by SDS-PAGE and visualized by autoradiography.

Membrane stained with Ponceau S is shown below as a loading control.

Figure 7. Replication elongation is required for cyclin E accumulation on chromatin.

Cycling LSS extracts were incubated with sperm DNA for the indicated times in the absence (lanes 1-6) or the presence (lanes 7, 8) of aphidicolin (Aphid, 40 $\mu\text{g/ml}$) before isolating chromatin templates by sedimentation and resolving chromatin-associated proteins by SDS-PAGE. Upper panels show Western blots for cyclin E and Cdc6, which remain bound to chromatin in varying amounts throughout DNA replication (DNA rep). Later time points showed no additional assembly of cyclin E onto chromatin in aphidicolin-treated samples. Lower panel shows IP kinase assays of samples identical to those above. Anti-cyclin B antibodies conjugated to protein A sepharose beads were used to immunoprecipitate cyclin B, and associated kinase activity was assayed by *in vitro* phosphorylation of histone H1 in the presence of [^{32}P - γ]-dATP. The peak in cyclin B kinase activity indicates that the extracts are in mitosis (M).

Figure 8. Specific mitotic kinases are capable of phosphorylating cyclin E and displacing cyclin E/Cdk2 from chromatin; Cdc14 can oppose phosphorylation by these kinases.

A. Sperm chromatin assembled in interphase LSS (in the presence of cycloheximide) for 1 hr was subsequently treated with buffer (lane 1), 1 unit of MAP-kinase (lane 2), cyclin B/Cdc2 (lane 3), Plk-1 (lane 4), or 10 μM okadaic acid (lane 5) for 10 min. Chromatin was extracted, associated proteins resolved by SDS-PAGE, and Western blotted for the presence of cyclin E or ORC.

B. Purified GST-Xcyclin E was incubated with buffer (lanes 1-2), MAP-kinase (lanes 3-4), cyclin B/Cdc2 (lanes 5-6), Plk1 (lanes 7-8), or cyclin E/Cdk2 (lane 9-10) in the presence of [^{32}P - γ]-dATP. After 30 min, 2 μM GST-Cdc14 was added to indicated

samples (lanes 2, 4, 6, 8, & 10) and all samples were incubated for a further 30 min. Reactions were resolved by SDS-PAGE and phosphorylated GST-cyclin E visualized by autoradiography.

Figure 9. Cdc14 reverses the inability of mitotic, hyperphosphorylated cyclin E to bind to chromatin.

A. Interphase extract (lanes 1-4) or mitotic extract stabilized by the addition of non-destructible cyclin B (lanes 5-8) was supplemented with buffer (lanes 1 & 4), 10 μ M okadaic acid (OA, lanes 2 & 6), 1 μ M GST-Cdc14 (lanes 3 & 7), or both OA and Cdc14 (lanes 4 & 8) and incubated at 23°C for 30 min. Reactions were stopped by adding sample buffer, proteins resolved by SDS-PAGE, and Western blots performed with cyclin E antibodies to detect the various phosphorylated forms of cyclin E.

B. Baculovirus expressed *Xenopus* cyclinE/Cdk2 in an auto-hyperphosphorylated form was mixed with buffer (lane 2), increasing concentrations of the CIP phosphatase (lanes 3-5), or increasing concentrations of GST-Cdc14 (lanes 6-8) for 30 min. In the upper panel, untreated HSS (lane 1) and treated samples were resolved by SDS-PAGE and analyzed by Western blotting with antibodies to cyclin E. In the lower panel, the samples in lanes 2-8 were incubated with λ DNA templates and a small amount of HSS. Assembled chromatin was isolated by sedimentation, and proteins were resolved by SDS-PAGE and analyzed by Western blotting with anti-cyclin E antibodies. The sample in lane 1 is HSS that was not treated (NT).

Figure 10. Model of the cell-cycle regulated association of cyclin E/Cdk2 with chromatin and its effects on DNA replication and re-replication control.

In a first phase, cyclin E/Cdk2 is recruited to origins of DNA replication by ORC, Cdc6, and possibly an unknown factor (denoted "X?"). In this conformation, with MCMs bound, DNA replication is initiated. In a second phase, dependent on the progression of

replication forks, multiple molecules of cyclin E accumulate on chromatin, blocking re-replication. In a final phase, cyclin E is hyperphosphorylated by cyclin B/Cdc2 and stripped from chromatin in mitosis. Rebinding of cyclin E to chromatin in interphase is possible only after dephosphorylation by Cdc14 or a related phosphatase. (See Discussion.)

Legend for Supplementary Figure

Supplementary Figure: N-terminal deletion mutants of Cdc6 fail to bind to cyclin E, to be phosphorylated by cyclin E/Cdk2, and to sustain replication in Cdc6-depleted extract.

A. LSS was immunodepleted with affinity purified Cdc6 antibodies conjugated to Protein A Sepharose beads. Depleted samples were supplemented with 80 nM of GST-tagged wild-type or N-terminally truncated XCdc6 as noted, prior to the addition of sperm DNA, an energy regenerating system, and [α - 32 P]-dCTP. Replication was quantitated as indicated in Materials and Methods and plotted as a percent of undepleted extract, normalizing the amount of replication in undepleted LSS to "100%." This corresponds to 1.5 ng/ μ l of new DNA synthesized from the 2.5 ng/ μ l of DNA added.

B. Purified GST (lane 2), wild-type GST-XCdc6 (lane 3), or the indicated N-terminal deletion mutant of GST-XCdc6 (lane 4-7) was incubated with radiolabeled IVT Xcyclin E (top panel) or ORC1 (middle panel). After a 30-min incubation, samples were diluted in IP buffer, GST-proteins were precipitated with glutathione agarose beads and washed. Beads were resuspended in sample buffer and associated proteins were resolved by SDS-PAGE and visualized by autoradiography. Lane 1 shows the amount of IVT protein used in each binding reaction. Membrane stained with Ponceau S is shown (bottom panel) as a loading control.

C. Purified GST (lane 1), GST fused to the N-terminal half of XCdc6 (lane 2), wild-type GST-XCdc6 (lane 3), or the indicated N-terminal truncation mutant of GST-XCdc6 (lane 4-7) was incubated with purified baculovirus expressed cyclin E/Cdk2 in the presence of [32 P- γ]-dATP (30 min, 23°C). Proteins were resolved by SDS-PAGE and phosphorylated proteins visualized by autoradiography.

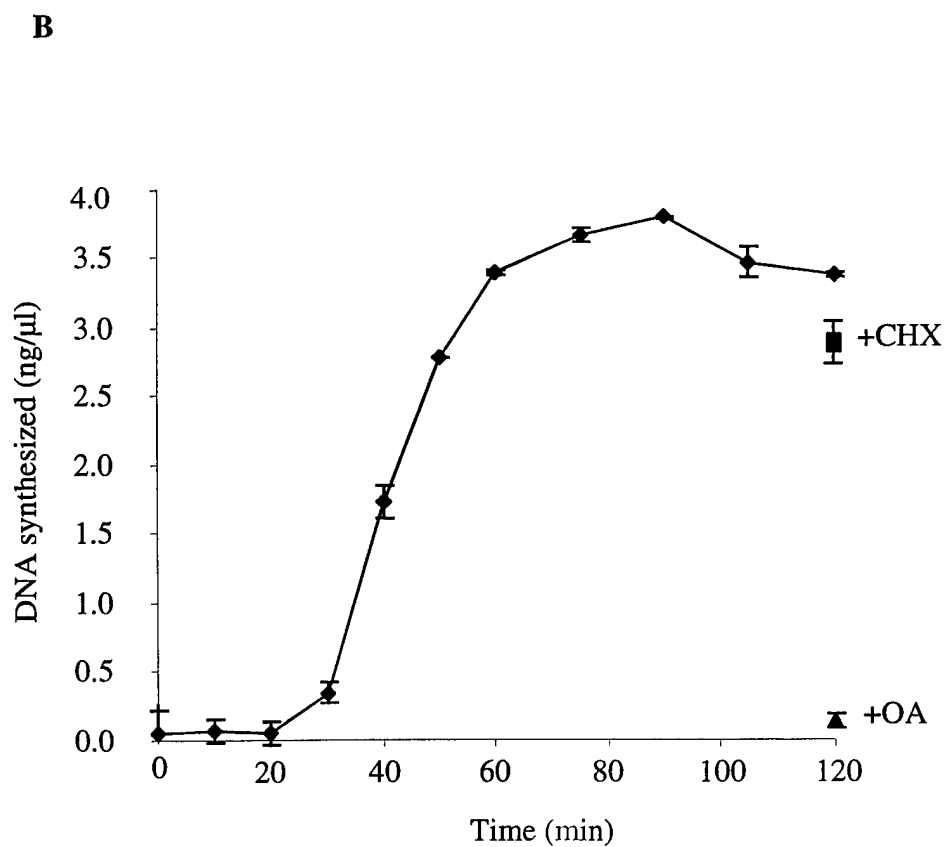
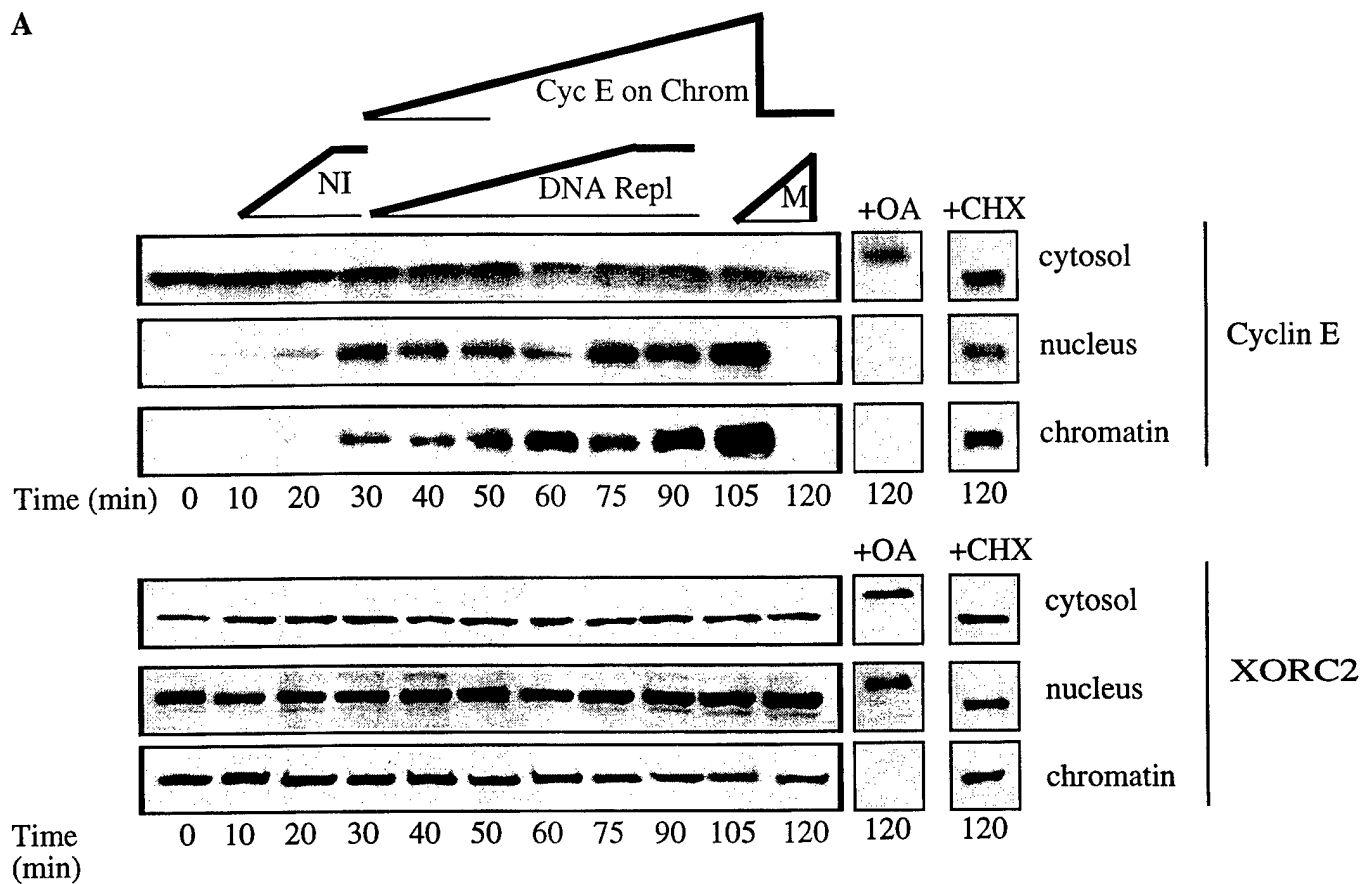


Figure 1, Furstenthal et al.

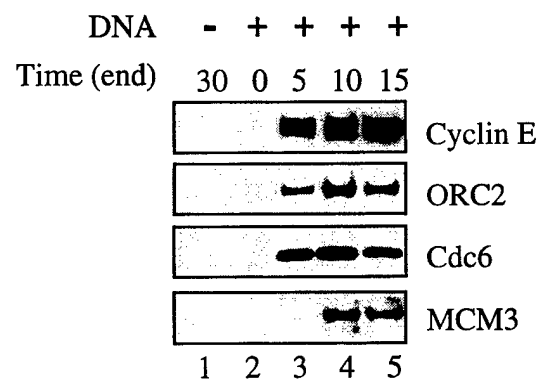


Figure 2, Furstenthal et al.

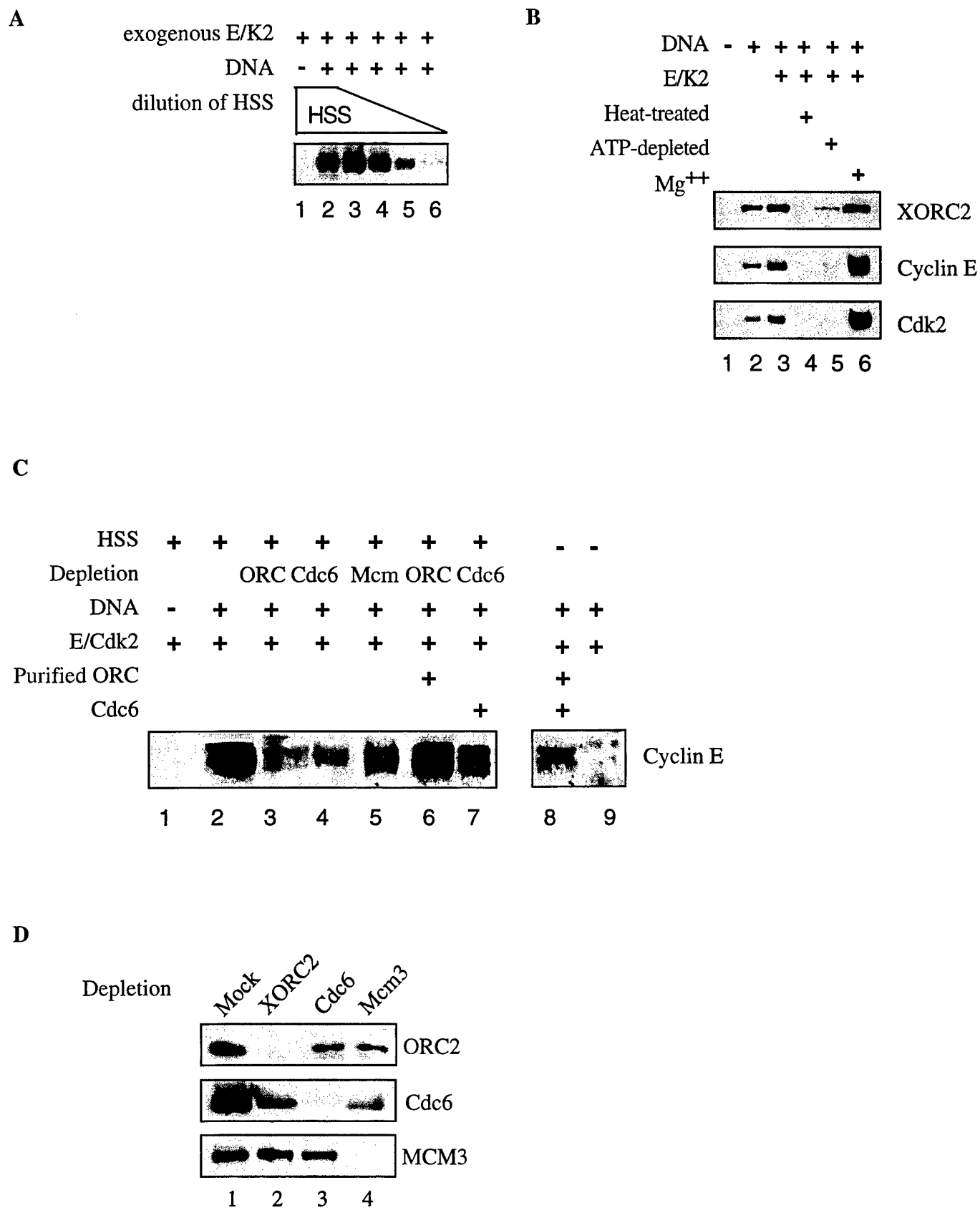


Figure 3, Furstenthal et al.

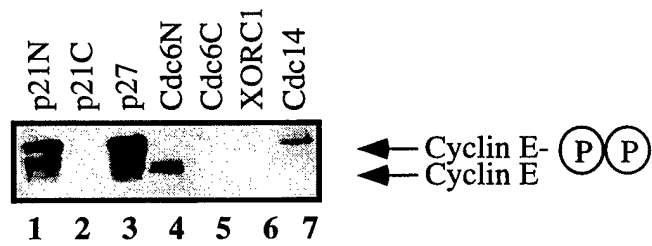


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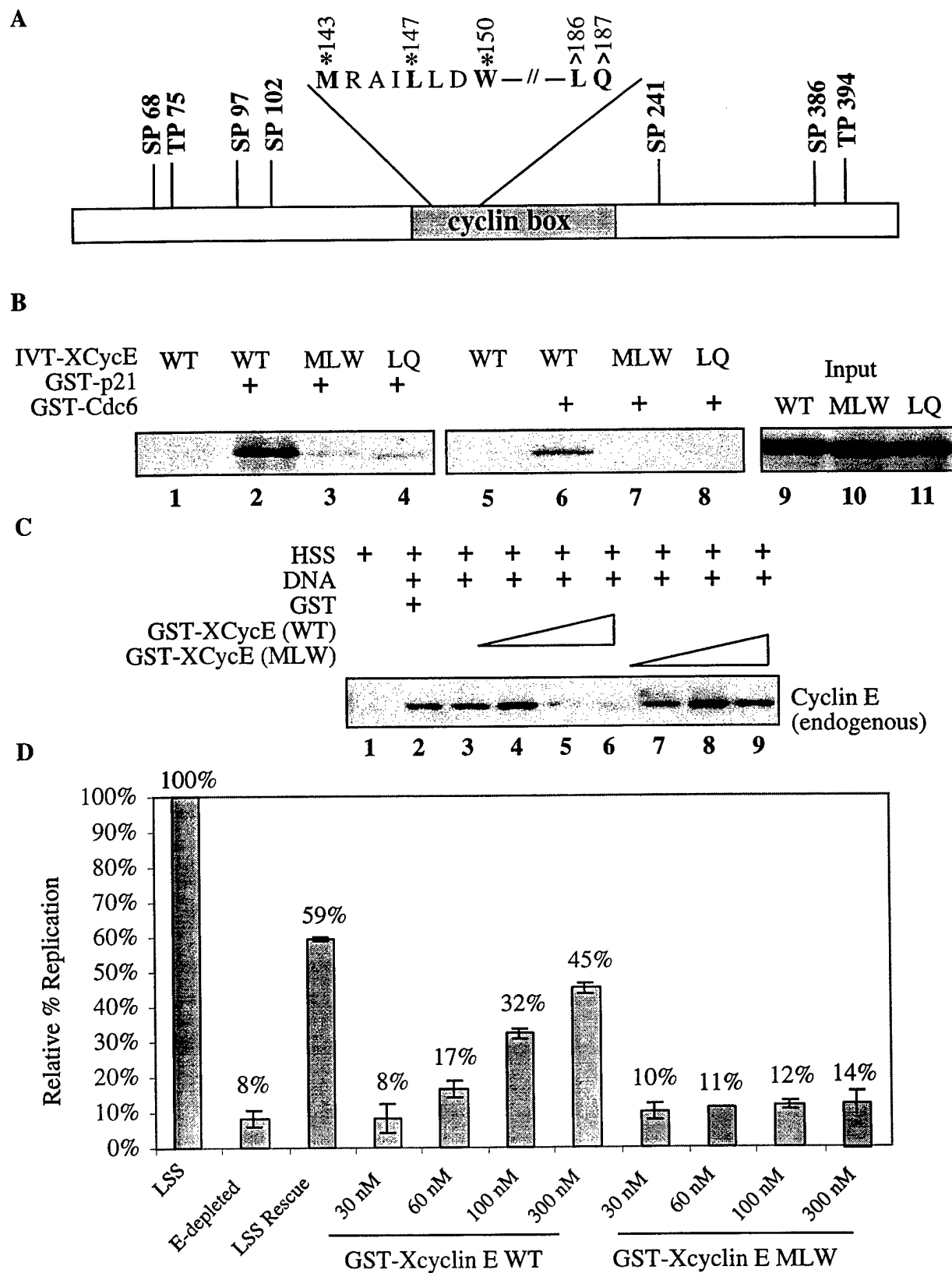


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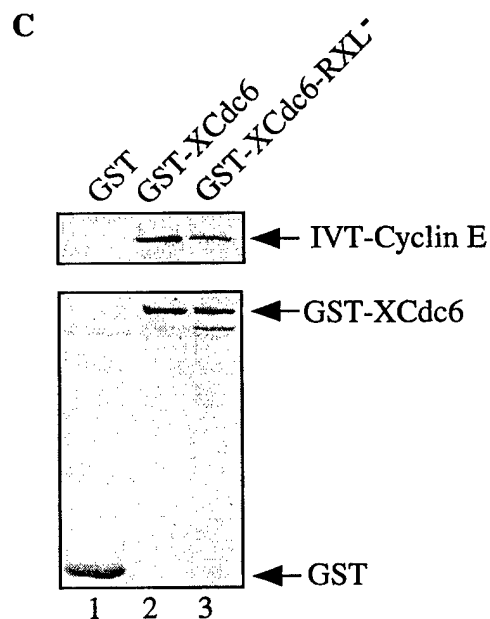
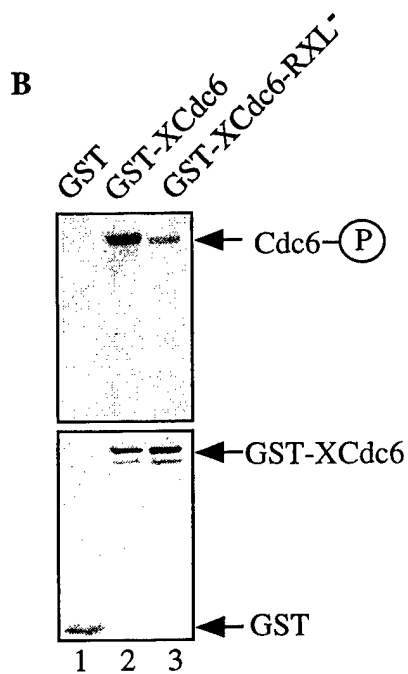
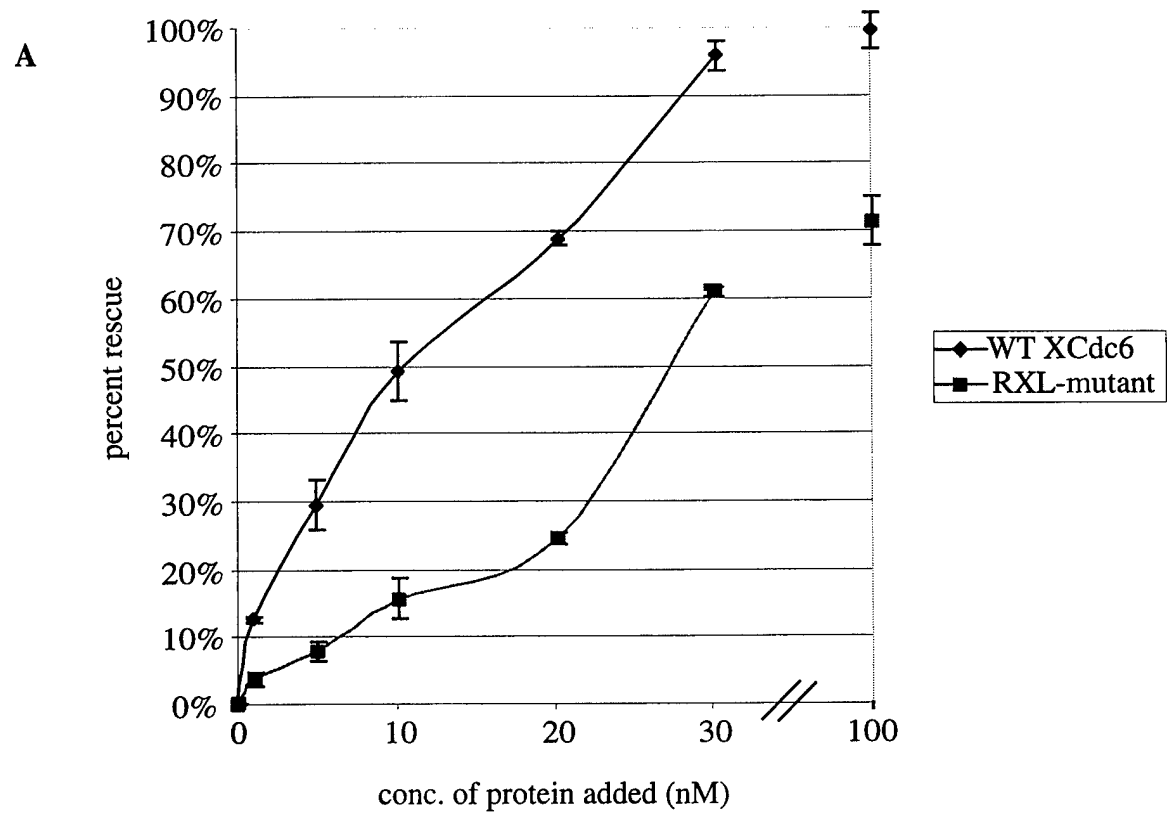


Figure 6, Furstenthal et al.

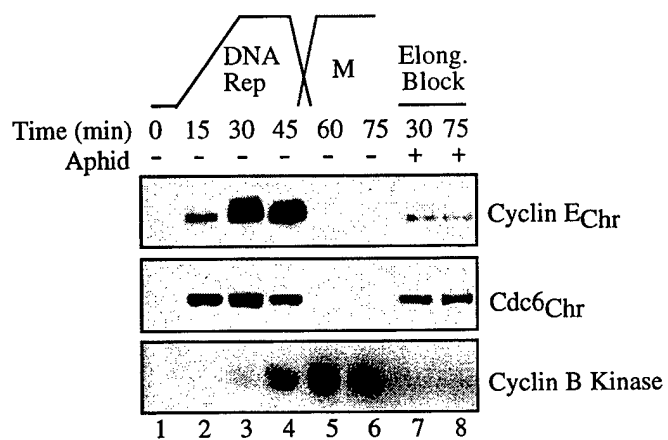
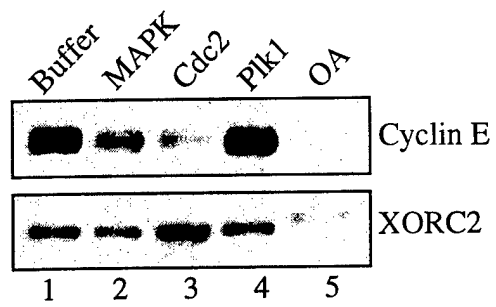


Figure 7, Furstenthal et al.

A



B

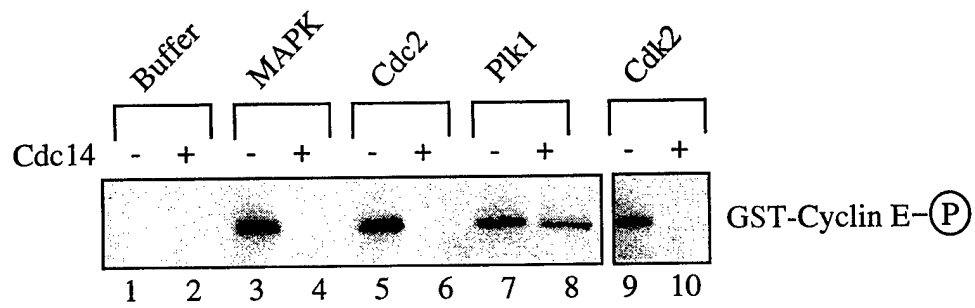
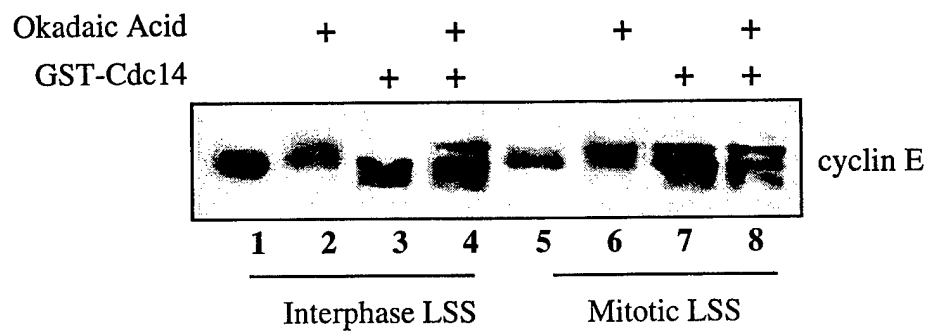


Figure 8, Furstenenthal et al.

A



B

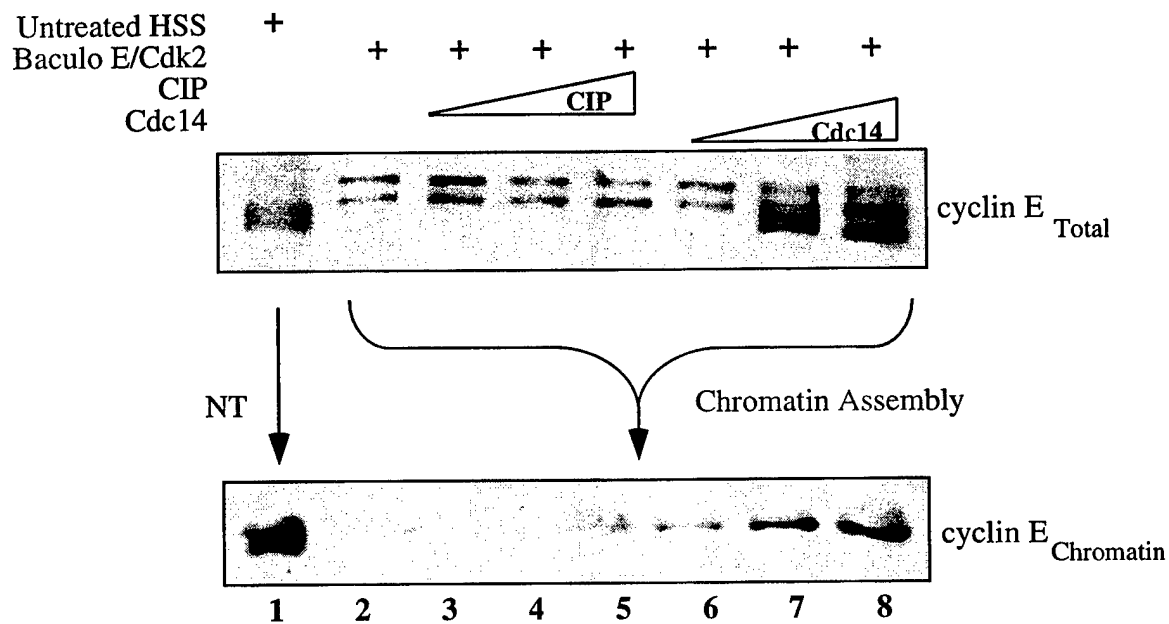


Figure 9, Furstenthal et al.

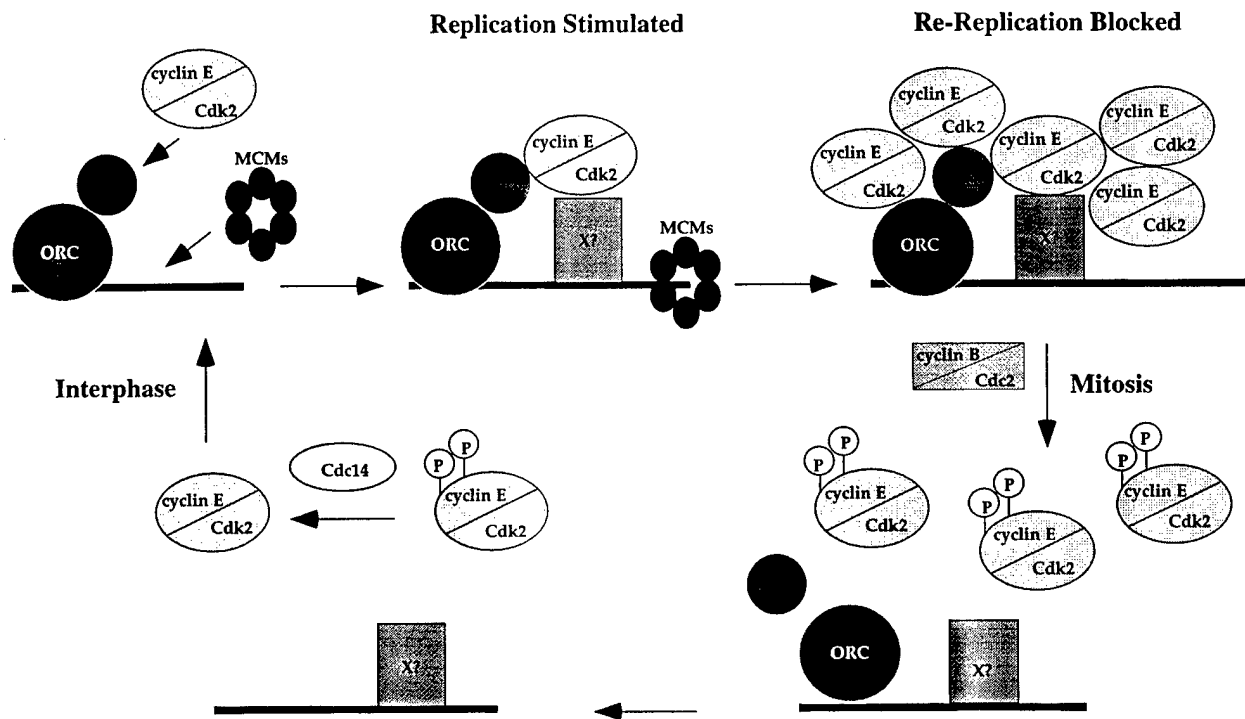
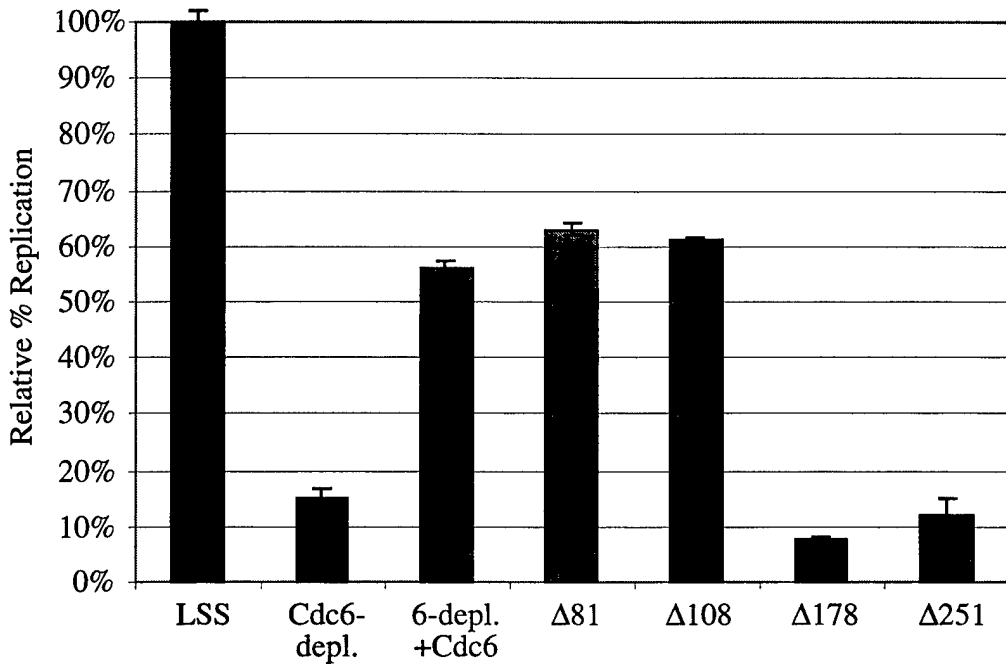
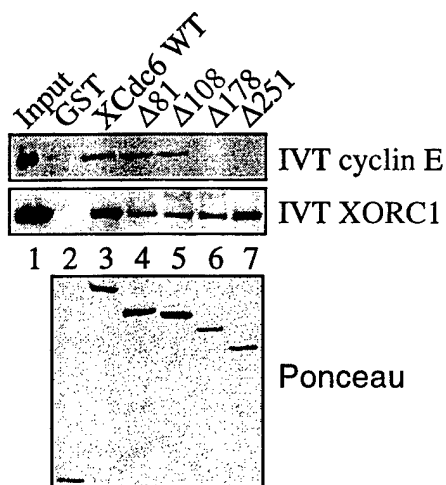


Figure 10, Furstenthal et al.

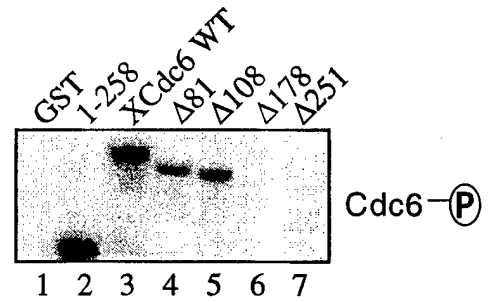
A



B



C



Supplementary Figure, Furstenthal et al.

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**Emi1 is a novel mitotic regulator that interacts with Cdc20 and inhibits
the Anaphase Promoting Complex**

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Summary

We have discovered a novel Early Mitotic Inhibitor, Emi1, which regulates mitosis in *Xenopus* embryos by inhibiting the Anaphase Promoting Complex/Cyclosome (APC). Emi1 is a conserved protein containing an F-box and an essential zinc-binding motif. Emi1 protein oscillates in the cell cycle and immunodepletion of Emi1 from cycling *Xenopus* extracts strongly delays cyclin B accumulation and mitotic entry, whereas expression of nondestructable Emi1 causes a prometaphase block. Emi1 binds Cdc20, and Cdc20 can rescue an Emi1-induced block to cyclin B destruction. The prophase to metaphase delay between cyclin B/Cdc2 activation and APC activation is critical for timing mitotic events but is not well understood. Our results suggest that Emi1 regulates the timing of mitosis by preventing premature APC activation by Cdc20, and may help explain the well-known delay between cyclin B/Cdc2 activation and cyclin B destruction.

Introduction

Mitotic entry is regulated by maturation promoting factor (MPF), a kinase complex composed of cyclin B and Cdc2 (Meijer et al., 1989; Minshull et al., 1989; Murray and Kirschner, 1989). Mitotic exit requires MPF inactivation, which is achieved by cyclin B destruction. Cyclin B is degraded through ubiquitin-dependent proteolysis, triggered by the Anaphase Promoting Complex/Cyclosome (APC) ubiquitin ligase (reviewed in Zachariae and Nasmyth, 1999). However, activated MPF must mediate nuclear envelope breakdown, chromatin condensation, and spindle formation before cyclin B is degraded. How this critical delay between MPF activation and APC activation is achieved remains unclear.

The vertebrate APC is composed of at least eleven subunits, including APC2, a member of the cullin family, and APC11, a RING-H2 finger protein (Gmachl et al., 2000; Yu et al., 1998). The APC shares homology with other ubiquitin ligases, including the SCF (Skp1, Cullin, and E-box protein) ubiquitin ligase, which contains a cullin and a RING-H2 finger protein as its catalytic core (Kamura et al., 1999; Seol et al., 1999; Skowyra et al., 1999; Tan et al., 1999). In the SCF, substrate recognition appears to be mediated by F-box proteins (reviewed in Jackson et al., 2000). In contrast, substrate recognition by the APC is less well understood.

Although present throughout the cell cycle, the APC is inactive while cyclin B accumulates in S, G2, and early M phase. APC activation is achieved through the binding of the WD-repeat containing proteins Cdc20 or Cdh1 (reviewed in Page and Hieter, 1999). In somatic cells, Cdc20 and Cdh1 binding to the APC is differentially regulated, resulting in a peak of APC^{Cdc20} activity in mitosis and APC^{Cdh1} activity in G₁ (Fang et al., 1998; Kramer et al., 2000; Rudner and Murray, 2000; Shirayama et al., 1998; Zachariae et al., 1998). In the *Xenopus* embryo however, Cdh1 is not expressed, and only APC^{Cdc20} is active (Kramer et al., 2000; Lorca et al., 1998). Cdc20 and Cdh1 may serve as substrate adapters for the APC similarly to the proposed function for F-box proteins in the SCF. However, there is as yet no evidence that Cdc20 or Cdh1 directly bind the proteins they target for destruction, and how they activate the APC is not understood.

The APC targets proteins containing a destruction (D) box or a KEN box motif for ubiquitin-mediated proteolysis (Pfleger and Kirschner, 2000). Nonetheless, the timing of APC substrate destruction is varied, suggesting that additional factors control the timing of APC activity. For example, cyclin A destruction begins in prometaphase, inhibitors of sister chromatid separation (securins) are destroyed at the metaphase-anaphase transition, and proteolysis of the mitotic kinase Plk1 and the spindle-associated protein Ase1 occurs as cells exit mitosis (Geley et al., submitted; Charles et al., 1998; Juang et al., 1997; Shirayama et al., 1998).

Phosphorylation by mitotically active kinases including cyclin B/Cdc2, Plk1, and PKA regulates APC activity in mitosis (reviewed in Page and Hieter, 1999; Zachariae and Nasmyth, 1999). Mitotic APC phosphorylation promotes its activation by Cdc20 (Kramer et al., 2000; Rudner and Murray, 2000; Shteinberg et al., 1999). However, mitotic APC phosphorylation may not be sufficient to explain the timing of APC activity, because APC from interphase extract can be activated *in vitro* by Cdc20 or Cdh1 (Fang et al., 1998; Kotani et al., 1999). Additionally, ectopic expression of Cdc20 or Cdh1 at any stage in the cell cycle activates the APC in yeast (Schwab et al., 1997; Visintin et al., 1997), suggesting that regulation is complex.

APC^{Cdc20} activity is also restrained by the spindle checkpoint (SC), a regulatory pathway conserved in yeast and vertebrates (reviewed in Chen and Murray, 1997; Straight and Murray, 1997). The SC protein Mad2 functions in prometaphase on unattached kinetochores to inhibit the APC until chromosomes align at the metaphase plate. Mad2 binds Cdc20 to inhibit APC activity (Fang et al., 1998; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998; Li et al., 1997; Wassmann and Benezra, 1998), although how Mad2 inhibits APC^{Cdc20} is not clear. The APC also localizes to centrosomes and the mitotic spindle (Tugendreich et al., 1995), where it may direct the local degradation of critical substrates (Clute and Pines, 1999; Huang and Raff, 1999).

We have identified a novel APC regulator called Emi1. Emi1 shares homology with the *Drosophila* protein Regulator of Cyclin A (Rca1), a positive regulator of cyclin A (Dong et al., 1997). Loss of Rca1 blocks embryos in G₂ of cell cycle 16. Rca1 overexpression in G₁ causes precocious cyclin A/Cdk2 activation by increasing cyclin A protein levels without affecting its transcription. How Rca1 mediates this effect is not known.

We show here that Emi1 promotes the stabilization of the mitotic cyclins by inhibiting APC activity. Emi1 accumulates in S phase and is destroyed in mitosis independent of the APC, but dependent on phosphorylation by Cdks. Emi1 overexpression blocks cells in mitosis with high cyclin B levels and inhibits cyclin B ubiquitylation *in vitro*. Emi1 binds Cdc20 directly and Cdc20 can rescue the Emi1-induced stabilization of cyclin B *in vitro*. Immunodepletion of Emi1 from egg extracts prevents the accumulation of cyclin B and mitotic entry. Collectively, our data indicate that Emi1 helps restrain APC activity through regulation of Cdc20.

Results

Xenopus Emi1 is a cell cycle regulated protein related to *Drosophila* Regulator of cyclin A (Rca1)

We initially isolated Emi1 in a yeast two-hybrid screen for proteins that interact with Skp1. Details of this screen are discussed elsewhere (Regan-Reimann et al., 1999). We cloned a full-length *Xenopus* Emi1 oocyte cDNA (see Experimental Procedures). The predicted Emi1 protein is 392 residues long and contains an F-box domain and a zinc binding motif, as well as five possible Cdk phosphorylation sites (Figures 1A and 1B). Additionally, there are two potential nuclear localization sequences (Figure 1A). BLAST search revealed that Emi1 has homology to the *Drosophila* protein Rca1 (Dong et al., 1997), (Figure 1A). Emi1 and Rca1 are similar in size, placement of functional domains, and share 25% similarity (16% identity). Emi1 is 43% similar (35% identical) to human Fbx5, a recently identified F-box protein of unknown function (Cenciarelli et al., 1999). Mutation or deletion of the Emi1 F-box abrogates binding to Skp1 *in vitro* (Figure 1B).

Xenopus Emi1 and its homologs contain 8 cysteines and a histidine in the C terminus that are highly conserved and may comprise two zinc binding domains (Figure 1A). The spacing of the cysteines and histidine in Emi1/Rca1, C-x(2)-C-x(14-30)-C-x(4)-C-x(4)-C-x(2)-C-x(4)-H-x(4)-C, is similar but not identical to the recently described

DRIL (TRIAD) cysteine-rich motif (van der Reijden et al., 1999). Thus, Emi1 may represent a novel zinc binding region (ZBR).

Affinity purified antibodies against *Xenopus* Emi1 recognize a protein of the expected molecular mass (44 kDa) in egg extracts and *Xenopus* XTC lysates (Figure 1C, left). The antibodies also recognize *in vitro* translated (IVT) Emi1, but fail to detect a protein in unprogrammed reticulocyte lysate. Pre-incubation of the antibodies with Emi1 protein blocks recognition of the 44 kDa species (Figure 1C, right).

Emi1 protein levels oscillate in a cell cycle-dependent manner

We examined the Emi1 protein in the cell cycle of the early embryo. In fertilized eggs, Emi1 levels increase in S phase and decrease in M phase (Figure 2A). Emi1 is present in CSF arrested eggs and persists after fertilization through the longer first interphase, during pronuclear migration.

Extracts made from activated eggs reproduce the events of the cell cycle *in vitro* (Lohka and Masui, 1983; Murray and Kirschner, 1989; Newport and Kirschner, 1984). Both endogenous Emi1 and exogenous IVT Emi1 added to these extracts are ubiquitinated in mitosis (figure 2B). Emi1 destruction requires the proteasome because IVT Emi1 is stabilized when the proteasome inhibitor MG-132 is added to mitotic egg extracts (Figure 2D), further confirming that Emi1 is destroyed in mitosis through ubiquitin-mediated proteolysis.

To assay whether Emi1 levels are also regulated in the somatic cell cycle, we arrested XTC cells in G₀ (serum starvation), early S (aphidicolin treatment), S (aphidicolin plus release), or M phase (nocodazole treatment). Emi1 protein levels are low in quiescent cells, highest in S phase cells, and decrease in mitosis (figure 2C).

Because Emi1 is mitotically destroyed, we tested whether it is an APC substrate. IVT Emi1 or an N-terminal cyclin B fragment was incubated in *Xenopus* egg extracts stabilized in mitosis by addition of non-destructable cyclin B ($\Delta 90$). In these $\Delta 90$ extracts, the APC is active and cyclin B is degraded (King et al., 1995). IVT Emi1 protein is destroyed in $\Delta 90$ extracts, showing that this system recapitulates Emi1 destruction requirements. APC immunodepletion or addition of a peptide containing the cyclin B destruction box, known to inhibit APC-mediated proteolysis (Holloway, 1993; King, 1995), prevented the destruction of cyclin B, whereas a control peptide did not (Figure 2E). However, Emi1 was destroyed with similar kinetics when the APC was depleted, blocked by destruction box peptides, or a control peptide (Figure 2E). Thus, Emi1 does not appear to be an APC substrate in the egg.

To investigate the sequence requirements for Emi1 destruction, we constructed Emi1 fragments containing only the N terminus or the C terminus (Figure 1B). IVT Emi1 N terminus (Emi1-NT) was destroyed with kinetics similar to full-length Emi1 in $\Delta 90$ extracts ($t_{1/2}$ ~10 minutes, whereas the C terminus (Emi1-CT) was stable ($t_{1/2}$ >100

minutes; Figure 2F). Because the N terminus contains four of the five possible Cdk phosphorylation sites in Emi1, we mutated serine or threonine to alanine in all five sites and found that this Emi1-5P mutant was stable in $\Delta 90$ extracts compared to wild type (Figure 2G). Interestingly, the N terminus of Emi1 identified cyclin B1 and B2 as interacting proteins several times in a yeast two hybrid screen (data not shown). We do not yet know whether Emi1 is a Cdk substrate *in vivo*, but we found that full-length Emi1 and Emi1-NT were efficient *in vitro* cyclin B/Cdc2 substrates, although neither the Emi1-CT nor Emi1-5P mutants were phosphorylated (Figure 2H). Further, Emi1 binds the mitotic cyclins A and B *in vitro* and Emi1 is a phospho-protein in egg extracts (data not shown). Thus, a plausible model is that phosphorylation of Emi1 by mitotically active kinases triggers the APC-independent destruction of Emi1.

Emi1 inhibits APC activity in *Xenopus* egg extracts

The oscillation of Emi1 in *Xenopus* embryos and the phenotype of Rca1-deficient *Drosophila* embryos, suggested that like cyclin B, Emi1 accumulation may be important for events leading up to mitosis and that Emi1 destruction may be necessary for mitotic exit. To test whether Emi1 destruction is important for mitotic exit, we analyzed the effect of Emi1 addition to *Xenopus* extracts. Addition of purified MBP-Emi1 fusion protein to cycling extracts prevented the destruction of endogenous cyclin B and mitotic exit (Figure 3A). Addition of equimolar amounts of MBP alone or another *Xenopus* F-

box protein had no effect on cyclin B stability or exit from mitosis (not shown). By quantitative immunoblotting, we estimate Emi1 to be ~300 nM in interphase egg extracts. As little as 100 nM additional Emi1 protein stabilizes cyclin B. However, we see a stronger effect on cyclin B stability with 300 nM to 1 μ M Emi1 protein concentrations, possibly because Emi1 is itself destroyed in mitosis.

To test whether Emi1 directly affects cyclin B ubiquitylation, we measured cyclin B ubiquitylation in Δ 90 extracts treated with purified MBP or MBP-Emi1 protein. Addition of MBP-Emi1 strongly reduced the ubiquitylation of an iodinated amino-terminal fragment of cyclin B containing the destruction box, whereas MBP did not (Figure 3B). Addition of purified MBP-Emi1 to APC immunoprecipitated from mitotic extracts also reduced APC *in vitro* activity compared to MBP alone (data not shown). Because Emi1 binds cyclin B (see previous section), we considered whether Emi1 specifically affects cyclin B ubiquitylation or also inhibits the destruction of other APC substrates such as *Xenopus* securin and geminin (McGarry and Kirschner, 1998; Zou et al., 1999). Both geminin and securin were stabilized by addition of Emi1 to Δ 90 extracts (Figure 3C). Unlike cyclin B, neither geminin nor securin appears to bind Emi1 (data not shown), indicating that interaction is not required for stabilization of APC substrates by Emi1.

We examined a series of Emi1 mutants to further dissect the domains of Emi1 required to block cyclin B destruction (see schematics, Figure 1B). Cyclin B was destroyed in $\Delta 90$ extracts treated with buffer alone (control) or an MBP-Emi1-NT fusion protein. In contrast, cyclin B was stabilized in the presence of MBP fusions to wild type Emi1, Emi1-5P, the F-box mutant (EL198AA), or Emi1-CT (Figures 3D and 3E). Therefore, the Cdk sites, the F-box, and the region N-terminal to the F-box are not required for Emi1 to stabilize cyclin B; however, the C terminus is both necessary and sufficient. An Emi1 truncation mutant missing the C-terminal ZBR (Emi1- Δ ZBR) was incapable of stabilizing cyclin B (Figure 3E). To further test the importance of the ZBR, we made single or double point mutations in the conserved ZBR cysteines. Mutation of either cysteine 341 or cysteine 346 to serine (mutants C341S and C346S respectively) greatly reduced the ability of Emi1 to inhibit cyclin B destruction (Figure 3F). Thus, the ZBR appears necessary for Emi1 to function as an APC inhibitor.

To test whether Emi1 affects the cell cycle *in vivo*, we injected the protein into one blastomere of a two-cell stage *Xenopus* embryo. Emi1 caused a stable cell cycle arrest in the injected blastomere, whereas the uninjected blastomere continued to divide normally (Figure 3G). Moreover, Emi1-blocked embryos at 2 1/2 hours had a high level of histone H1 kinase activity similar to that detected in $\Delta 90$ extracts, whereas uninjected and control-injected embryos had H1 kinase levels similar to interphase extracts (Figure

3G). As in cycling extracts, the Emi1 C-terminus with an intact ZBR was also necessary and sufficient to mediate the mitotic block *in vivo* and wild type and N terminal Emi1 are unstable *in vivo* (Figure 3G and data not shown).

Overexpression of Emi1 in somatic cells causes a prometaphase block

To examine Emi1 subcellular localization, we stained *Xenopus* XTC cells with affinity purified antibodies to Emi1. In interphase, we found the protein localizes in a punctate pattern in the nucleus and the cytoplasm, with some perinuclear concentration (Figure 4A). In mitotic cells, Emi1 localized throughout the cell and particularly at the spindle (Figure 4A and B).

To determine more precisely at what point in mitosis Emi1 blocks, we overexpressed epitope-tagged variants of Emi1 in somatic cells. Because Emi1 is unstable in mitotic XTC cells (data not shown), the myc-tagged Emi1 variants were cotransfected with a GFP expression construct to mark transfected cells. Transfection of wild type Emi1, EL198AA, Emi1-5P, or Emi1-CT caused an increase in the mitotic index compared to vector alone, whereas neither Emi1-NT nor the C346S point mutant had a significant effect (Figures 4C). This was confirmed by flow cytometric analysis of DNA content (Figure 4E). Overexpression of the well-known APC inhibitor Mad2 in XTC cells caused a quantitatively similar strong increase in mitotic index as wild-type Emi1 (data not shown). Examination of DNA and spindle morphology revealed that cells

transfected with Emi1, EL198AA, Emi1-5P, or Emi1-CT specifically accumulated in prometaphase or metaphase (Figure 4D). In summary, Emi1 blocks the cell cycle at mitosis both *in vitro* and *in vivo* and prevents the ubiquitin-mediated destruction of known APC substrates *in vitro*.

Depletion of Emi1 prevents cyclin B accumulation and entry into mitosis

If Emi1 normally inhibits APC activity in interphase, then depletion of Emi1 from cycling egg extracts might block cyclin B accumulation, preventing mitotic entry.

Following Emi1 immunodepletion (Figure 5D), we examined cyclin B accumulation and DNA morphology as markers of mitotic entry. In control cycling extracts, cyclin B protein peaks by 80 minutes and is destroyed by 120 minutes. In contrast, cyclin B levels fail to accumulate in Emi1-depleted extracts (Figure 5A). Addition of beads from the Emi1 immunodepletion rescued the accumulation and subsequent destruction of cyclin B. Addition of purified Emi1 protein rescued the accumulation of cyclin B but blocked its destruction (Figure 5A). This is likely because excess Emi1 may not be completely destroyed, thus inhibiting the APC and stabilizing cyclin B.

The effects of Emi1 depletion on cell cycle progression were verified by observing DNA morphology in cycling extracts. In control extracts, demembranated sperm DNA was highly condensed by 60 minutes, indicating onset of mitosis, and typically displayed anaphase or telophase morphology by 90 minutes (Figure 5B and C).

DNA in Emi1-depleted extracts maintained a decondensed interphase morphology (Figure 5B and C). Addition of undepleted extract or purified Emi1 protein to the depleted extracts rescued mitotic entry. Although Emi1-depleted extracts rescued with undepleted extract progressed past metaphase, extracts rescued with Emi1 protein did not. APC activity is also required to destroy securin and allow sister chromatid separation (reviewed in Nasmyth et al., 2000). Because Emi1 inhibits securin destruction *in vitro* (Figure 3C), excess Emi1 may prevent chromosome segregation by this mechanism.

If Emi1 depletion prematurely activates the APC, then addition of the APC inhibitor Mad2 should also rescue mitotic entry. Addition of Mad2 to Emi1-depleted extracts did rescue mitotic entry (Figure 5B and C) although as in the case of the rescue with Emi1 protein, the extracts did not progress beyond metaphase. To test whether the inability of Emi1-depleted extracts to enter mitosis was primarily due to their inability to accumulate cyclin B, we also tested whether addition of $\Delta 90$ cyclin B rescued mitotic entry. $\Delta 90$ addition to depleted extracts rescued nuclear envelope breakdown and mitotic DNA condensation, indicating that nondestructable cyclin B can overcome the requirement for Emi1 in these events (Figures 5B and 5C).

Cdc20 can rescue cyclin B destruction in the presence of Emi1

Because Emi1 represented a new cell cycle regulator, we looked for interacting proteins by yeast two hybrid screens of a *Xenopus* oocyte library using Emi1 as a probe.

Screening with full-length Emi1 identified only Skp1, therefore we tested Emi1-NT and Emi1-CT for interacting proteins as well (see Experimental Procedures). As previously mentioned, Emi1-NT isolated cyclin B.

Importantly, Emi1-NT also identified the APC activator Cdc20. To validate this interaction, we took several approaches. First, interphase extracts resolved on anion exchange and gel filtration columns showed that Emi1 and Cdc20 fractions overlap (data not shown). Specifically, Cdc20 co-immunoprecipitates with Emi1 from a fraction spanning 100-140kDa (Figure 6A). The APC-Cdc20 complex is approximately 1500 kDa in *Xenopus* extracts (Fang et al., 1998; King et al., 1995), but this fraction contains no Emi1. Emi1 also does not appear to precipitate the APC components Cdc27 and APC2 from extracts (data not shown). Second, we reconstituted the interaction between Emi1 and Cdc20 using baculovirus-expressed proteins (Figure 6B). Third, we found that purified Emi1 can bind purified Cdc20 (Figure 6C). We knew that the N terminus of Emi1 interacts with Cdc20 from our two hybrid screen, but the C terminus of Emi1 (Emi1-CT) also binds Cdc20 *in vitro* (Figure 6C). We also confirmed this interaction in the yeast two hybrid system. Interestingly, we also observed both in yeast two hybrid binding and in *in vitro* binding assays, that the N terminus, but not the WD repeat domain of Cdc20, is required for binding to Emi1 (data not shown).

If Emi1 inhibits Cdc20 activation of the APC, then Cdc20 should rescue the Emi1 block to cyclin B destruction. Addition of baculovirus-expressed Cdc20 to mitotic extracts rescued the Emi1-induced block to cyclin B destruction in a dose-dependent manner (Figure 6D), supporting the hypothesis that Emi1 prevents Cdc20 from activating the APC.

Discussion

We have identified and characterized a novel APC inhibitor called Emi1, which is required for mitotic entry. Emi1 is unstable in mitosis and expression of non-destructable versions of the protein or overexpression of the wild type protein causes a mitotic block in both embryos and somatic cells. Emi1 destruction is APC-independent in the egg and likely requires phosphorylation by Cdks. Much like the APC itself, Emi1 localizes to the mitotic spindle, which may contribute to its ability to prevent destruction of APC substrates. The APC activator Cdc20 binds to Emi1 and can rescue the Emi1-induced block of cyclin B degradation, indicating that Cdc20 may be the target of Emi1-APC regulation.

Identification of an independent cell cycle oscillator which controls APC activity

Emi1 is destroyed in mitosis by ubiquitin-mediated proteolysis and its destruction likely requires phosphorylation by mitotic kinases, including cyclin B/Cdc2. We do not know the mechanism of Emi1 destruction in mitosis, but it does not require the APC. Several F-

box proteins are unstable in mitosis, and the SCF has been implicated in their destruction. Additionally, SCF substrates identified to date are regulated by phosphorylation (reviewed in Jackson et al., 2000). Thus, mitotically phosphorylated Emi1 may be an SCF substrate, although what triggers Emi1 destruction remains to be determined.

Emi1 destruction may also be influenced by its association with Cdc20 or the mitotic spindle. An interesting possibility is that phosphorylation by cyclin B triggers the dissociation of Emi1 and Cdc20 or removes Emi1 from the mitotic spindle, thereby promoting Emi1 destruction. Indeed, we found that Cdc20 addition not only rescued the Emi1 block of APC activity, but also stabilized Emi1 in mitotic extracts (JDRR, PKJ, unpublished data) suggesting that Emi1 is more stable when complexed with Cdc20.

Does Emi1 participate in a novel checkpoint for APC activation?

Cdc20 exists in high molecular weight complexes both with and independent of the APC (Kramer et al., 1998; Lorca et al., 1998). Emi1 and Cdc20 co-immunoprecipitate from interphase extracts in a complex independent of the APC, suggesting a model where Emi1 sequesters Cdc20 from the APC (see model, Figure 7). Another possibility is that Cdc20 is a substrate of an Emi1-containing SCF ubiquitin ligase complex. We do not yet know if Emi1 is associated with E3 activity, but addition of Emi1 to egg extracts does not destabilize Cdc20 (JDRR, PKJ, unpublished data), making it unlikely that Emi1 directs Cdc20 destruction. Further, the F-box is not required for Emi1 to block cyclin B

destruction. Cdc20 is also stable in the early embryo (Kramer et al., 2000; Lorca et al., 1998) so the ability of Emi1 to regulate the cell cycle does not require Cdc20 destruction. Further, in yeast and somatic cells, Cdc20 is an APC^{Cdh1} substrate (Pfleger and Kirschner, 2000; Prinz et al., 1998; Shirayama et al., 1998).

Our rescue experiments suggest that Emi1 is a direct inhibitor of Cdc20. The ZBR of Emi1 is required to inhibit the APC, although how it affects APC activity is not clear. One possibility is that this region cooperates with the N terminus of Emi1 to bind Cdc20, a hypothesis supported by preliminary *in vitro* binding data with Emi1 C terminal fragments and Cdc20 (JDRR, PKJ, unpublished data).

Cyclin B ubiquitylation activity of APC immunoprecipitated from synchronized HeLa cells increases significantly before cyclin B levels decrease, and the APC subunit Cdc27 is phosphorylated well before cyclin B levels decrease (Kramer et al., 2000). This delay in APC activation even when the APC is phosphorylated by MPF suggests the presence of an inhibitor that restrains full APC activation until nuclear envelope breakdown, spindle assembly, and chromatin condensation have occurred. The delay might be explained in part by Mad2, which is required for APC inhibition in prometaphase until chromosomes have been properly aligned at the metaphase plate (Gorbsky et al., 1998; Taylor and McKeon, 1997). However, even though Mad2 is thought to broadly inhibit APC^{Cdc20} when activated by unattached kinetochores, the range

of Mad2 signal may not be sufficient to control the APC throughout the cell. Also, although anti-Mad2 antibody injection affects progression through metaphase, it does not affect progression through prophase, when MPF is also active and Cdc20 is present (Gorbsky et al., 1998).

It is possible that Emi1 cooperates with Mad2 to inhibit Cdc20 and is thus part of the SC pathway. However, Emi1 does not bind to Mad2 *in vitro* (JDRR, PKJ, unpublished data) and Emi1 binds to Cdc20 directly *in vitro*. Additionally, unlike Mad2 and other SC proteins, Emi1 does not appear to localize to kinetochores (EF, JDRR, PKJ, unpublished data). Further, cyclin A destruction is not inhibited when Mad2 is activated (Waizenegger et al, 2000; Geley et al, submitted), whereas addition of Emi1 to cycling extracts stabilizes endogenous cyclin A (JDRR, PKJ, unpublished data), suggesting that Emi1 can also control cyclin A destruction. Cyclin A normally gets destroyed before cyclin B in mitosis (Minshull et al., 1990), indicating that Emi1 may inhibit the APC early in mitosis, before APC inhibition by Mad2.

The observation that Emi1 immunodepletion delays cyclin B accumulation and mitotic entry further indicates that Emi1 may inhibit the APC prior to and in early mitosis, before Mad2 begins to function. Additionally, loss of the putative *Drosophila* Emi1 homolog Rca1 prevents mitotic entry (Dong et al., 1997), similar to our observation that Emi1 is required for mitotic entry in egg extracts. Blocking the proteasome with

MG-132 also rescued cyclin B accumulation in Emi1-depleted extracts (JDRR, PKJ, unpublished data), further indicating that Emi1 depletion affects cyclin B stability rather than, for example, its translation. Moreover, addition of the APC inhibitor Mad2 to depleted extracts also rescues mitotic entry, indicating that Emi1 depletion triggers APC activation. However, we cannot exclude that Emi1 may have additional roles for promoting mitotic entry.

APC activation is spatially as well as temporally regulated. Recent studies indicate cyclin B proteolysis begins first at the spindle poles (Clute and Pines, 1999; Huang and Raff, 1999). Might there be sensing mechanisms other than Mad2 and the SC that regulate the APC? Interestingly, the recently identified Chfr protein has been implicated in a checkpoint that delays metaphase entry in response to mitotic stress (Scolnick and Halazonetis, 2000). Chfr may monitor proper completion of centrosome separation. Mitotic events other than centrosome separation and kinetochore capture by microtubules, namely nuclear envelope breakdown, spindle formation, and chromatin condensation, must occur sequentially. It is therefore possible that these critical prophase events are controlled by sensing mechanisms that involve Emi1.

Experimental Procedures

Cloning of Emi1 and Emi1 yeast two hybrid screen

A partial cDNA isolated from a yeast two hybrid screen with Skp1 (Regan-Reimann et al., 1999) was used to screen a *Xenopus* ovary cDNA library (Stratagene). The longest of 4 independent clones isolated (1.88 kb) was sequenced on both strands and contains stop codons upstream of the putative 5' start codon and a 3' poly-A tail. *In vitro* transcription of the clone produces a 44 kDa species in reticulocyte lysate.

Emi1-NT (1-193), Emi1-CT (233-392), or Emi1 full-length (fl) were cloned into pAS2 (Clontech) and used to screen a *Xenopus* oocyte library (Clontech) in the yeast strain Y190 (~2.5 million independent clones screened per construct). Interacting proteins were verified with fl Emi1 by filter lift β -galactosidase assay, with lamin and p53 proteins as negative controls.

Preparation of full-length, deletion and point mutant constructs, and proteins

Wild type (wt) Emi1 and variants were cloned into pCS2-5mt (myc-tagged). Site-directed mutagenesis of wt Emi1-pCS2-5mt was performed to create point mutants: E198A and L199A (EL198AA), S10A, S29A, S105A, T123A, S328A (Emi1-5P), C341S, C346S, C354S and C356S (C354S/C356S), and C364S. Mutations were verified by sequencing. Wt Emi1 baculovirus was generated using the BAC-TO-BAC system (Gibco), in SF9 cells.

Both MBP-Emi1 and Emi1 cleaved from MBP behave similarly in egg extracts. For egg extract experiments, Emi1 variants were cloned into pMAL-c2 and MBP fusion proteins were produced and purified by standard protocols.

Human Cdc20 baculovirus expression construct (E. Kramer) was expressed in High-Five cells (Invitrogen), extracts prepared as in (Kramer et al., 2000), cells lysed in a cell disruption bomb (Parr Instrument Co.), and protein purified using Ni-NTA agarose (Qiagen).

Antibody preparation

Bacterially produced MBP-Emi1 was used to raise polyclonal antibodies in rabbits and mice (Josman laboratories). Rabbit antibodies were affinity purified on a GST-Emi1 column. Both affinity purified rabbit anti-Emi1 and anti-Emi1 mouse polyclonal sera recognize endogenous and overexpressed Emi1 on immunoblots.

Binding assays and chromatography

In vitro GST-Skp1 binding reactions were performed as in (Bai et al., 1996).

For *in vitro* MBP fusion protein binding assays, 100 nM purified MBP-Emi1, MBP-Emi1-NT, MBP-Emi1-CT, or MBP was incubated with 100 nM His-Cdc20 in Buffer 1 (50mM Tris pH7.5, 100mM NaCl, 0.1% NP-40), and supernatants incubated with amylose resin. Beads were washed 4X with buffer 2 (2 (50 mM Tris pH7.5, 300 mM

NaCl, 1% NP-40), and bound proteins resolved by SDS-PAGE and anti-MBP immunoblots (NEB).

For baculovirus reconstitution assay, SF9 cells co-infected with Emi1 and Cdc20 baculoviruses were lysed in RIPB (100 mM NaCl, 50 mM beta-glycerophosphate, 5 mM EDTA, 0.1% triton-X 100, 1 mM DTT, protease inhibitors), and lysates pre-cleared with protein G sepharose. Supernatants were incubated with polyclonal mouse anti-Emi1 sera or preimmune sera, bound to protein G sepharose, washed 4X in RIPB, and bound proteins resolved by SDS-PAGE and anti-Cdc20 immunoblots.

High speed supernatant was prepared from interphase *Xenopus* egg extracts as in (Murray et al., 1989) then resolved on a Resource Q column by FPLC (Pharmacia). Bound proteins were eluted with a linear NaCl gradient (0- 0.5 M). Fractions containing Emi1 were pooled and resolved on an S-300 gel filtration column. The 100-140 kDa fraction was pre-cleared with protein G sepharose, incubated with polyclonal mouse anti-Emi1 sera or preimmune sera, bound to protein G sepharose, washed 4X in RIPB, and bound proteins resolved by SDS-PAGE and anti-Cdc20 immunoblots.

Phosphorylation reactions and kinase assays

Histone H1 kinase activity was assayed as in (Murray, 1991). Cyclin B kinase activity was assayed as in (Jackson et al., 1995).

For *in vitro* cyclin B phosphorylation experiments, 1 μ M purified MBP-Emi1 or MBP-Emi1 variants were incubated with 2 units cyclin B/Cdc2 (NEB) in kinase buffer plus 66 μ M ATP and 0.25 μ Ci/ μ l [32 P- γ]-ATP) (15min, RT). Reactions were quenched with sample buffer and resolved by SDS-PAGE.

***Xenopus* extracts and embryos**

Interphase extracts and cycling extracts were prepared as in (Murray, 1991), except that egg activation was performed with 0.5 μ g/ml calcium ionophore A23187 (Sigma). Sperm nuclei were prepared as described (Jackson et al., 1995). To assay DNA morphology in cycling extracts, nuclei were added (3000 sperm/ μ l extract), fixed at various times, and DNA labeled with Hoechst 33258 (Murray, 1991). Endogenous cyclin B levels in cycling extracts were assayed by immunoblotting with anti-Xl cyclin B2 mouse monoclonal antibodies (S. Geley, T. Hunt). Mitotic Δ 90 extracts were made by adding a bacterially expressed GST fusion of a nondegradable Δ 90 fragment of sea urchin cyclin B to interphase extracts at a concentration of 60 μ g/ml.

Xenopus eggs were fertilized *in vitro* (Murray, 1991). 10 eggs were isolated per time point, lysed in RIPB, and assayed for cyclin B-associated kinase activity and for Emi1 protein levels by immunoblotting. For embryo injection experiments, 9.2 nl of 100 μ M protein was injected into one blastomere at the two cell stage. Injected embryos were transferred to 0.1X MMR with 3% Ficoll. To assay H1 kinase activity, both blastomeres

in two-cell stage embryos were injected, 4 injected embryos pooled, and a histone H1 kinase assay was performed.

Degradation and ubiquitylation assays

The cyclin B substrate used was an N terminal sea urchin cyclin B fragment (aa 13-91) fused to protein A (Glutzer et al., 1991). To assay substrate degradation in $\Delta 90$ extracts or cycling extracts, ^{35}S -labeled IVT protein was added and extracts incubated (23°C).

Aliquots were removed, quenched in sample buffer, resolved by SDS-PAGE, and quantitated on a phosphorimager. Extracts were treated with 1 mM human cyclin B destruction box peptide or a scrambled version (E.Kramer), or depleted of the APC with anti-Cdc27 antisera to assay the effect of APC inhibition on Emi1 stability. Extracts were treated with 2 mM MG-132 to test the effect of proteasome inhibition on Emi1 stability. To assay the affect of Emi1 on APC substrate stability, 1 μM MBP fusion protein, 1 μM control protein, or buffer alone was added.

To assay Emi1's effect on cyclin B ubiquitylation, 2.5 μM MBP-Emi1 or MBP was incubated in $\Delta 90$ extracts (20 min), with 3ug/mL iodinated sea-urchin cyclin-B fragment (aa 13-110) plus 2 mg/ml ubiquitin, 0.4 mg/mL LLnL, and an energy regenerating system. Reactions were incubated at 25°C, aliquots removed, and analyzed by SDS-PAGE and phosphorimaging.

Immunodepletions

Anti-Emi1 rabbit polyclonal sera or preimmune sera were covalently coupled to protein-A coated affiprep beads (Bio-Rad). Beads were washed 5X with XB- (20 mM HEPES pH 7.7, 100 mM KCl), incubated with cycling extracts at 0.3ul beads/ul extracts (45 min, 4°C) with gentle rocking. Samples were spun (3 min, 3000 rpm, 4°C) and the process repeated 2 more times for 30 min at 4°C with gentle rocking, and the triple-depleted extracts put at 23°C to cycle. To rescue, depleted extracts were pre-incubated with 0.2 volumes undepleted extract, 300 nM MBP-Emi1, 0.3ul depletion beads/ul extract, 100 ng/ul Δ90, or 0.3 mg/ml GST-Mad2 (10 min, 4°C) prior to cycling.

Tissue culture, immunofluorescence and flow cytometry

Xenopus XTC cells were maintained as described (Freed et al., 1999) and blocked in the cell cycle as follows: serum starvation [72 h in medium with 0.5% FBS], nocodazole [0.1 μg/ml, 24 h], aphidicolin [cells were serum-starved (48 h), then incubated in normal medium plus aphidicolin (2 μg/ml, 30 h)]. Cells recovered from aphidicolin were washed and incubated in normal medium (4 h) before fixation or lysis. Blocks were confirmed by flow cytometric analysis.

Transfections were performed using Fugene 6 reagent (Roche Molecular Biochemicals). pEGFP-C1 (Clontech), and myc-Emi1 constructs were co-transfected (1:10). Under these conditions, 98% of GFP-positive interphase cells were also myc-

labeled. Cells were processed for immunofluorescence or flow cytometry 72h post-transfection.

For immunofluorescence, cells were grown on cover slips, washed in PBS and fixed in methanol (-20°C). Cells cotransfected with GFP plasmid were fixed in 2% paraformaldehyde. Cover slips were washed in Immunofluorescence Wash Buffer (IFWB: PBS/0.1% Triton X-100/3% BSA), and blocked in IFWB with 5% normal donkey serum. 1° Antibodies were used as follows: affinity-purified anti-Emi1 (1.5 µg/ml); anti- α -tubulin (Serotec rat anti- α -tubulin mAb, Clone YL1/2 supernatant; 1:1); anti-myc mAb 9E10 (1 µg/ml). [Cells fixed in paraformaldehyde yielded similar results to those fixed in methanol upon labeling with anti-Emi1 antibodies]. Texas Red or fluorescein-conjugated donkey 2° antibodies (Jackson ImmunoResearch) were used at 1:150, and Hoechst dye at 5 µg/ml. Fluorescent cells were visualized and digitally imaged as in (Freed et al., 1999). Deconvolution was performed as in (Freed et al., 1999).

Propidium iodide staining for DNA content and flow cytometric analysis were performed by standard methods, using a Beckman Coulter ALTRA flow cytometer. Cell cycle distribution was quantified using MultiCycle AV software (Phoenix Flow Systems, Inc.)

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Figure legends

Figure 1. Emi1 is a novel F-box protein related to *Drosophila* Rca1.

- A. Alignment of Emi1 with putative homologs. Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*. The alignment was performed using the CLUSTAL W method. Black= identity, dark gray= highly conserved changes, and light gray= less highly conserved changes. The F-box, putative zinc-binding region (ZBR), and putative NLS sequences are boxed.
- B. Schematic and key features of the wild-type Emi1 protein (Accession # AF319594) and mutant proteins used in this study (left). Emi1= wild type protein; EL198AA= site specific amino acid substitutions in 2 conserved F-box residues; Emi1-N terminus (NT)= amino acids 1-193; Emi1-C terminus (CT)= amino acids 248-392; Emi1-ΔZBR= amino acids 1-338; Emi1-5P= site specific substitution of alanine for serine or threonine in all five SP/TP sites; C346S= substitution of cysteine 346 with serine. GST-Skp1 or GST was incubated with ³⁵S-labeled *in vitro* translated (IVT) proteins and then bound to glutathione agarose. Proteins retained on beads after washing were analyzed by SDS-PAGE and autoradiography (right).
- C. Characterization of Emi1 antibody. Rabbit reticulocyte lysate (RRL) programmed with Emi1 (lane 1), unprogrammed RRL (lane 2), *Xenopus* XTC cell lysate (lane 3), and interphase *Xenopus* egg extract (lane 4) were resolved by SDS-PAGE and immunoblotted

with affinity purified anti-Emi1 (left) or affinity purified anti-Emi1 blocked with MBP-Emi1 protein (right).

Figure 2- Emi1p is destroyed early in mitosis

A. Emi1 levels fluctuate in the early embryonic cell cycle. Fertilized eggs were incubated at 23° C, equal numbers of embryos removed at the indicated times, lysed, and processed for immunoblotting with affinity purified anti-Emi1 antibody (upper panel) and for histone H1 kinase activity by IP kinase assay with anti-cyclin B1 antisera (lower panel). [H1 kinase activity increases as eggs enter mitosis and decrease as they exit mitosis.] We determined that the upper band seen on Emi1 blots of egg extract (Emi1-P) is a phosphorylated form of Emi1 (data not shown).

B. Emi1 is ubiquitylated in cycling extracts. Activated *Xenopus* cycling egg extracts with (left) or without (right) the addition of ³⁵S-labeled IVT Emi1 was incubated at 23°. Aliquots were removed at the indicated times, resolved by SDS-PAGE, and visualized on a phosphorimager (left panel) or immunoblotted with anti-Emi1 (right panel). I= interphase, M=mitosis, as determined by cyclin B ubiquitylation and Histone H1 kinase activity.

C. Emi1 destruction requires the proteasome. ³⁵S-labeled IVT Emi1 was added to *Xenopus* egg extracts stabilized in mitosis with nondestructable cyclin B protein (Δ90

extracts). These extracts were treated with either buffer alone (control) or 2 mM MG-132 (+MG-132). Aliquots were removed at the indicated times and resolved by SDS-PAGE.

D. Emi1 levels fluctuate in the somatic cell. XTC cells were grown asynchronously (lane 1), or blocked in the cell cycle by serum starvation (lane 2), aphidicolin (lane 3), aphidicolin plus release for 4 hours (lane 4), or nocodazole (lane 5). Samples were processed for immunoblotting with affinity-purified rabbit anti-Emi1 (upper panel) or *Xenopus* Orc1 as a loading control (lower panel).

E. Emi1 destruction does not require the APC. ³⁵S-labeled IVT N-terminal cyclin B fragment or wild type Emi1 was added to Δ90 extracts. These extracts were treated with either a destruction box (D-box) peptide, scrambled D-box peptide (control), or depleted of the APC with anti-Cdc27 antibodies. Aliquots were removed at the indicated times and resolved by SDS-PAGE.

F. Emi1 and its N-terminus are unstable, whereas the C terminus is stable, in mitotic extracts. ³⁵S-labeled IVT full-length, N-terminal, or C-terminal Emi1 was added to Δ90 extracts and assayed for stability as in E.

G. Mutation of the five putative Cdk phosphorylation sites stabilizes Emi1.

³⁵S-labeled IVT wild type Emi1 or a mutant in all five SP/TP sites (Emi1-5P) was added to Δ90 extracts and assayed for stability as in E.

H. Full-length and N-terminal Emi1 are phosphorylated by cyclin B/Cdc2 *in vitro*.

Equimolar amounts of purified MBP-Emi1, MBP-Emi1-NT, MBP-Emi1-CT, or MBP-Emi1-5P (Coomassie Blue counterstain not shown) were incubated with purified cyclin B/Cdc2 in the presence of [^{32}P]- γ -dATP. Proteins were resolved by SDS-PAGE and visualized by autoradiography.

Figure 3 - Emi1p prevents the ubiquitin-mediated destruction of APC substrates and inhibits exit from mitosis

A. Emi1p prevents cyclin B destruction and mitotic exit in cycling egg extracts.

Activated *Xenopus* cycling egg extracts were incubated with either buffer alone (■) or 1 μM purified MBP-Emi1 protein (Δ) in the presence (graph) or absence (lower panel) of sperm. Aliquots were removed at the indicated times and assayed for DNA morphology (graph) or processed for immunoblotting with antibodies to *Xenopus* cyclin B (lower panel). M= mitosis.

B. Emi1p inhibits cyclin B ubiquitylation by the APC. An ^{125}I -labeled cyclin B N-terminal fragment was incubated in $\Delta 90$ extracts treated with 2.5 μM purified MBP (left) or MBP-Emi1 (right). Aliquots were removed at the indicated times and resolved by SDS-PAGE. [The reduction in ubiquitin conjugates over time (left) is likely due to the presence of de-ubiquitylating enzymes in the extract.]

C. Securin and Geminin are stabilized in the presence of Emi1. ³⁵S-labeled IVT *Xenopus* securin or geminin was incubated in Δ90 extracts treated with either buffer alone (control) or 1 μM purified MBP-Emi1 protein. Aliquots were removed at the indicated times and resolved by SDS-PAGE.

D. The Emi1 C-terminus is sufficient to block cyclin B destruction. ³⁵S-labeled IVT cyclin B N-terminal fragment was added to Δ90 extracts treated with buffer alone (■) or 1 μM purified MBP-Emi1 (●), MBP-Emi1-NT (Δ), or MBP-Emi1-CT (□). Aliquots were removed at the indicated times, resolved by SDS-PAGE, and quantified on a phosphorimager.

E. The presence of the Emi1 ZBR but not the F-box domain is required to block destruction of cyclin B. ³⁵S-labeled IVT cyclin B N-terminal fragment was added to Δ90 extracts treated with buffer alone (■), or 1 μM purified MBP-Emi1 (●), MBP-EL198AA (▲), MBP-Emi1ΔZBR (□), or MBP-Emi1-5P (○). Aliquots were removed at the indicated times, and analyzed as in D.

F. Mutation of cysteine 341 or 346 to serine prevents Emi1 from inhibiting cyclin B destruction. ³⁵S-labeled IVT cyclin B N-terminal fragment was added to Δ90 extracts treated with buffer alone (■) or 1 μM purified MBP-Emi1 (●), MBP-C341S (▲), MBP-C346S (○), MBP-C351S (Δ), MBP-C354S/C356S (□), or MBP-C364S (◆). Aliquots were removed at the indicated times, and analyzed as in D.

G. Injection of full-length or the Emi1 C-terminus blocks *Xenopus* embryos in mitosis with high cyclin/Cdk kinase activity. 1 pmole purified MBP-Emi1, MBP-Emi1-NT, MBP-Emi1-CT, or MBP was injected into one blastomere (right side) of two-cell stage *Xenopus* embryos. Embryos were harvested and photographed 2.5h after injection (left panel). For kinase assays, two-cell stage embryos were injected in both blastomeres with MBP-Emi1 or MBP protein and harvested 2.5 hrs post-injection. Extracts from the injected embryos were assayed for cyclin/Cdk kinase activity by histone H1 kinase assays (right panel). Unfertilized eggs and equivalent aliquots of interphase and $\Delta 90$ extracts were assayed as controls.

Figure 4- Transfection of Emi1 into XTC cells causes a prometaphase-like block

A. Emi1 localization. XTC cells were labeled with affinity-purified antibodies to Emi1, anti- α -tubulin, and Hoechst 33258 dye. Anti-Emi1 antibodies were blocked with MBP-Emi1 protein ("Block"). The Emi1 staining (*red*) and α -tubulin (*green*) images were merged (*Merge*) to show the Emi1 spindle localization.

B. Deconvolution image of Emi1 spindle localization. XTC cells were labeled as in A, and the Emi1 staining (*red*) and α -tubulin (*green*) images were merged (*Merge*) to show the Emi1 spindle localization.

C. Overexpression of Emi1 causes an increase in mitotic index. XTC cells were co-transfected with GFP and myc-tagged constructs expressing Emi1, Emi1-NT, Emi1-CT,

EL198AA, C346S, or Emi1-5P. Cells were fixed and stained with anti- α -tubulin antibody and Hoechst 33258 dye. The number of mitotic GFP positive cells was quantitated based on DNA and spindle morphology.

D. Emi1 overexpression causes a prometaphase/metaphase block. XTC cells were co-transfected as in C. Cells were fixed, stained, and the number of GFP positive cells in each mitotic phase was quantitated as in C.

E. Flow cytometric analysis of Emi1-transfected XTC cells. Cells were fixed, labeled with propidium iodide, and analyzed by flow cytometry. Histograms depict the cell cycle profiles for GFP-positive cells for each transfection. Vector alone=1; Emi1=2; Emi1-NT=3; Emi1-CT= 4; Emi1 Δ ZBR= 5; C346S= 6; EL198AA= 7; Emi1-5P= 8. Table (right side) lists the % GFP positive cells in each cell cycle stage for each transfection. *[The percentage mitotic for the Emi1-5P mutant is likely an underestimate because many cells expressing this mutant undergo apoptosis.]

Figure 5. Loss of Emi1 prevents mitotic entry in egg extracts

A. Emi1 depletion prevents cyclin B accumulation in *Xenopus* cycling extracts. Equal aliquots were removed at the indicated times from preimmune-depleted, Emi1-depleted, or Emi1-depleted cycling extracts pre-incubated (10 min) with either 300 nM MBP-Emi1, extract (0.13 volumes), or beads from the Emi1 depletion. Samples were processed

for immunoblotting with anti-cyclin B2 and anti-Orc1 antibodies (as a loading control).

The exposure time is the same for all blots.

B. Emi1-depleted cycling extracts fail to enter mitosis. DNA was added to preimmune serum-depleted, Emi1-depleted, or Emi1-depleted cycling extracts pre-incubated (10 min) with either 300 nM MBP-Emi1, extract (0.2 volumes), 6 μ M Gst-Mad2, or 60 μ g/ml GST- Δ 90 cyclin B. Aliquots were removed at the indicated times, fixed onto slides, and DNA visualized by Hoechst 33258 staining.

C. Quantitation of mitotic figures. DNA was added to preimmune-depleted (■), Emi1-depleted (●), or Emi1-depleted extracts supplemented with either 300 nM MBP-Emi1 (○), extract (0.2 volumes, Δ), 6 μ M GST-Mad2 (□), or 60 ng/ul GST- Δ 90 cyclin B (◆).

Aliquots were removed at the indicated times and fixed onto slides. DNA morphology visualized with Hoechst 33258 staining and the number of interphase and mitotic figures was quantitated.

D. Equal amounts of undepleted, preimmune sera-depleted and Emi1-depleted extracts were resolved by SDS-PAGE and processed for immunoblotting with anti-Emi1 antibodies to show the efficiency of depletion. Emi1 depletion is estimated to remove ~80% of the protein.

Figure 6. Emi1p interacts with Cdc20 and Cdc20 rescues an Emi1-induced block to cyclin B destruction

A. Emi1 coprecipitates with Cdc20 from egg extracts. An Emi1-Cdc20 complex in interphase egg extracts was partially purified by chromatography on Resource Q anion exchange and S-300 gel filtration columns. Preimmune (PI) or anti-Emi1 immunoprecipitates from a 141kDa-100kDa fraction were assayed by immunoblotting with anti-Cdc20.

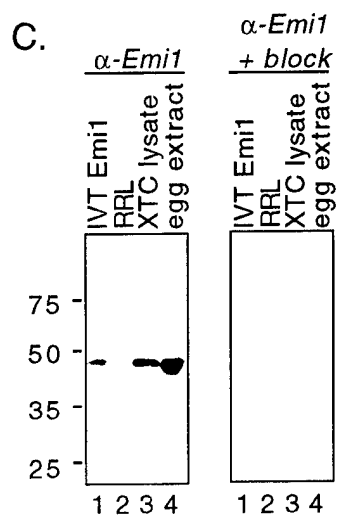
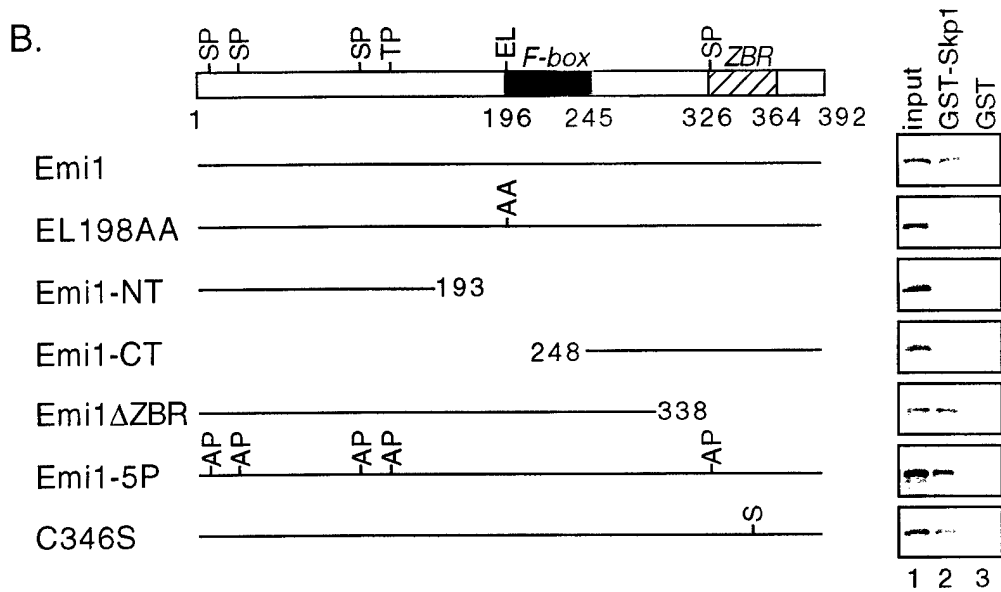
B. Emi1 and Cdc20 associate in baculovirus co-infection. SF9 cells were co-infected with baculovirus-expressed Emi1 and Cdc20, precipitated with preimmune or anti-Emi1 antisera, and assayed for the presence of Cdc20 by immunoblotting.

C. Cdc20 directly interacts with both the N-and C-terminus of Emi1 *in vitro*. Purified bacterially expressed MBP-Emi1 protein and purified baculovirus-expressed his-Cdc20 were incubated together in binding buffer and precipitated on amylose beads. Beads were washed and assayed for Cdc20 by immunoblotting.

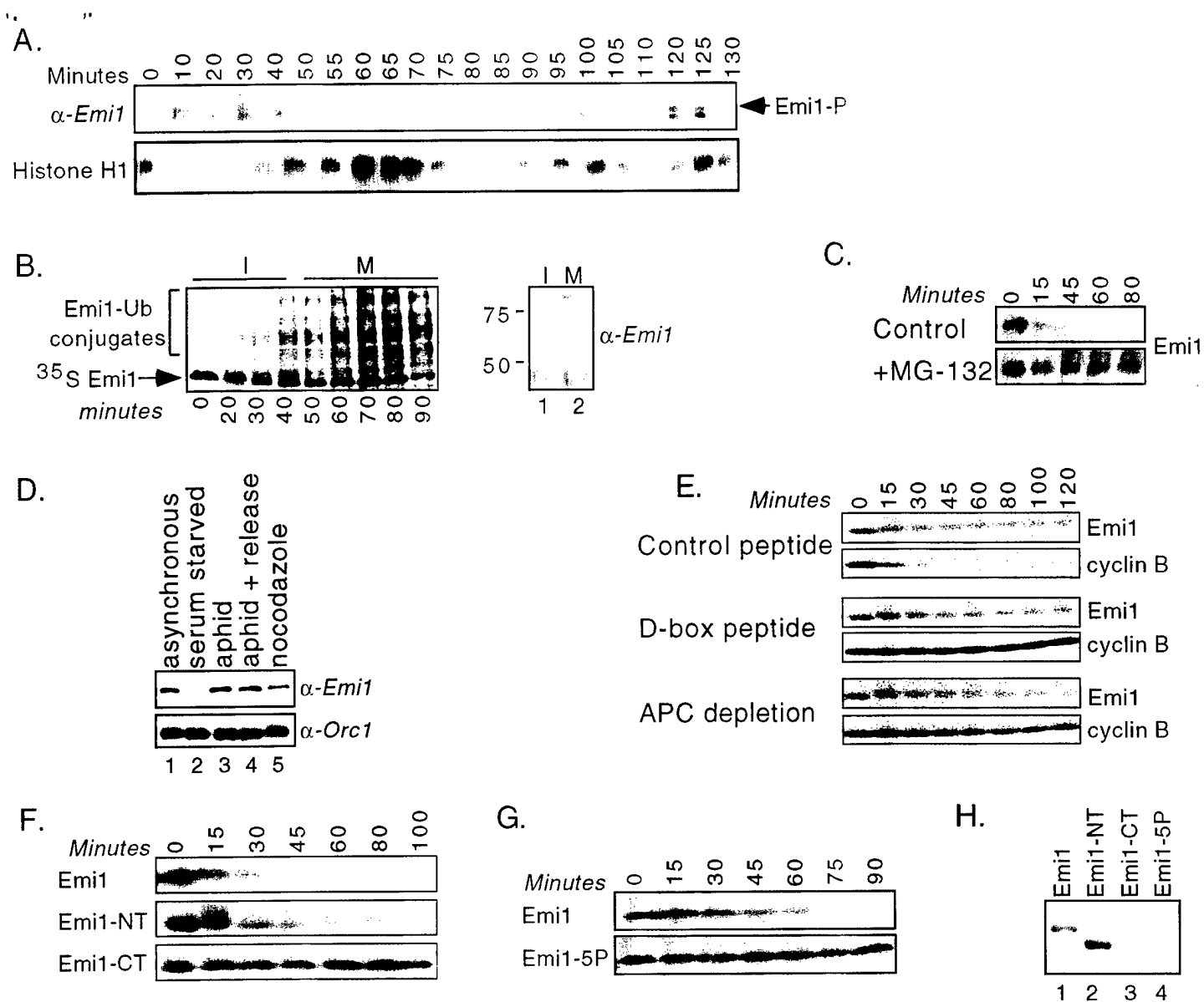
D. Cdc20p rescues cyclin B destruction. ³⁵S-labeled IVT N-terminal cyclin B was added to mitotic *Xenopus* egg extracts treated with buffer alone (■), 1 μM purified MBP-Emi1 (●), 1 μM MBP-Emi1 plus 1 μM his-Cdc20 (Δ), or 1 μM MBP-Emi1 plus 3 μM his-Cdc20 (□). Aliquots were removed at the indicated times, resolved by SDS-PAGE (left), and quantitated on a phosphorimager (graph).

Figure 7 - A model for the role of Emi1 in Anaphase Promoting Complex regulation.

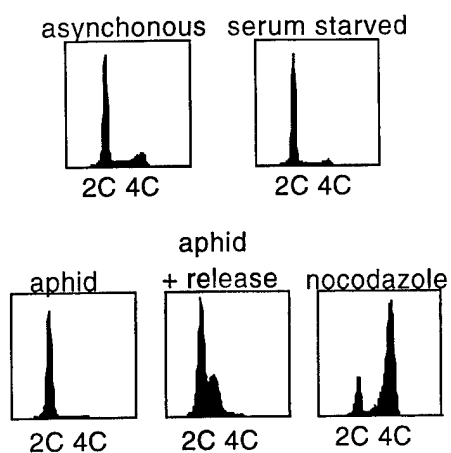
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Mm Emil	MSRRPCSCALRPPRCSCSASPSAVTAAGRPRPSDSCKEESSTLSVKMKDNCNHHVHSGKLVDPDDIGRLVS	64
Dm Rcal	MSRRPCSCALRPPRCSCSASPSAVTAAGRPRPSDSCKEESSTLSVKMKDNCNHHVHSGKLVDPDDIGRLVS	20
Xl Emil	ISPPVKKHPPCK--VYENVQSSCHDSACTTVAKCADLTDDLPVH--EN--LHR--NDL--TNSYEEY--AQ--D	95
Hs Emil	YTPAYLEGSCCKDCIKDYERISCHGSP--RIVQLETESKRLH--ENOHVQQT--NSTEIEAT--TSRIYED	145
Mm Emil	YSPVCLPESCNDCLRNRHERISFDSP--UGH--D--ENQVQNT--DSS--ETEEL--ASRIYED	124
Dm Rcal	-----SGYTSF--LHNSTA--ETPFLLEDAEGENC--NASNTT--FFRGLNTPSGHQED--	78
Xl Emil	GYSSI---LNDSPCQDETDRKVS--IQVRETPKNFMSYGRFHTLSKIN--LP--RFE--AGSTT--MRKTNRK	164
Hs Emil	GYSSFS---LQSGL--SEHEGSLLENFGDSLQSCLLQIQSPDQYPN--NL--LP--HFEKV--GSTT--KNAKRNPK	214
Mm Emil	GYSSF---TOSDR---DDGILILENFRNSPOARLLPS--SDQH--PN--TL--LP--HFEKV--GSTT--KNAKRNPK	189
Dm Rcal	LYWGKPYPRTPQOKKFSAE--EPFSMTPLQDEHS-----LPKR--KKHF--QSPHSSP--KSKK	133
Xl Emil	LDWNAV--DVVCGGNYG--EH--CKSK--G--BRFDI--A--LFH--DFKH--LTKILRH--A--D--L--VISVSTTWK--L--OK	236
Hs Emil	VDREM--KE--IARGNR--R--ON--GRK--C--ECVDI--S--LFR--GLRH--LATILAQ--D--D--L--V--SVSTTWK--L--ED	287
Mm Emil	VDREM--KE--IARGNR--R--ON--GRK--C--ECVDI--S--LFR--GLRH--LATILAQ--D--D--L--V--SVSTTWK--L--ED	262
Dm Rcal	LLFPHE--EPPKNRF--G-----G--EKL--DI--AKLAQW--Q--PALQCILRHVGAHTLDVMTKVS--PAW--KQAVYR	195
Xl Emil	DNWAAVNAVKLGCKELCEKRAKVSSHTATRDESLC-----V--LASVOKVA-----A--SLC--S--K--SKNKN	296
Hs Emil	DKGA--POLYSKAIQ--R--T--F--NNK--F--SPH--AST--REYVMF-----T--ELASVOKSA-----A--QTS--L--K--DAQT--L	345
Mm Emil	NKGA--POLYSKTMQ--R--V--I--SS--K--L--SLH--AT--RGYVVG-----A--ALTCVOKSS-----T--WAPP--K--KDVQ--K	319
Dm Rcal	SQRDLE--RLQNHREKLNLT--KENP--P--KRC--SHV--P--ANHT--L--QTS--NHSS--LANSVRLANGLGQ--L--EHPPD--GRGCR	266
Xl Emil	G-----GLSCNR-----LAEFIEV--O--KND--SL-----KVCVDCGSP--KHDPCL-----	336
Hs Emil	SNQGDQKGSTYSR-----HNEFSEV--K--K--KNE--SL-----KACIRCNS--P--AYDCYL-----	391
Mm Emil	SSQRGQRVSTYSR-----HNEFVEV--K--K--KNE--SL-----KACVRCNF--E--AKYDHYL-----	305
Dm Rcal	KGAARRDACC--K--C--PRCG--G--SRV--F--L--S--B--K--CG--EN--LSQTLPIGRTTSTFP--CMT--GPPL--KRFL--SLDLDEVRTSPQG	339
Xl Emil	---H--A--I--CT--RESCKLD--C--C--CK--H--FS--S--C--MS--P--G--SYRIP--S--E--L--G--S-----K--K--Q--N--L--R--R--L--	392
Hs Emil	---Q--A--T--CK--REGCGED--C--C--CK--H--HT--T--DC--SD--G--L--L--ASCKIG--L--E--G--T-----K--K--K--K--L--R--R--L--	447
Mm Emil	---E--A--V--CK--RESCKED--C--C--CK--H--H--N--N--D--C--NG--I--L--ASCKVG--L--E--G--T-----K--K--K--K--L--R--L--	421
Dm Rcal	PPYNFAE--CTSVICQ--R--E--C--V--N--C--CK--S--H--P--G--E--R--C--V--T--E--L--D--T--P--S--K--L--M--P--R--E--R--L--T--P--P--Q--R--A--Q--N--R--D--P--K--I--T--R--K--N--S--L--K--R--L--C--F	412

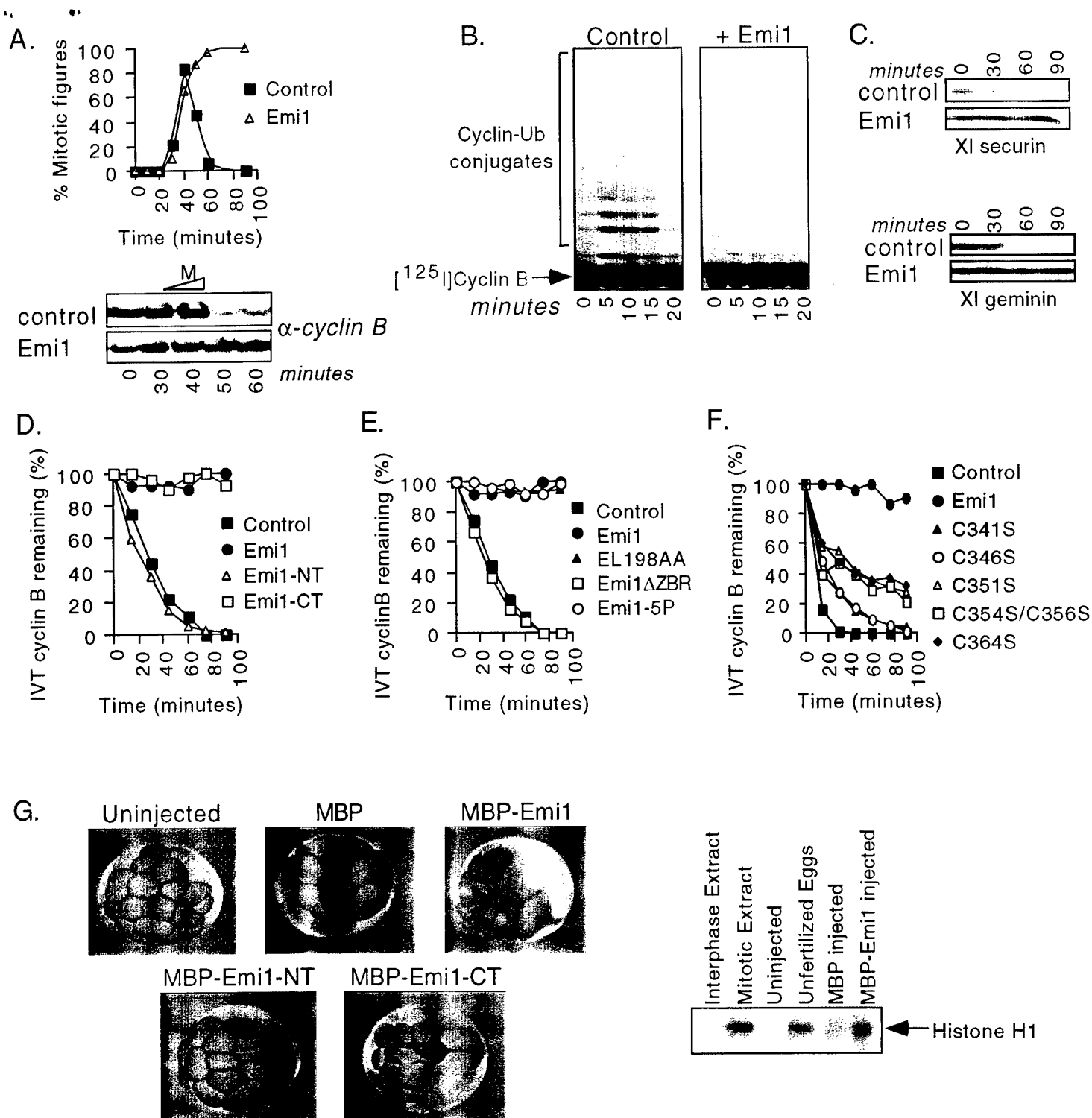


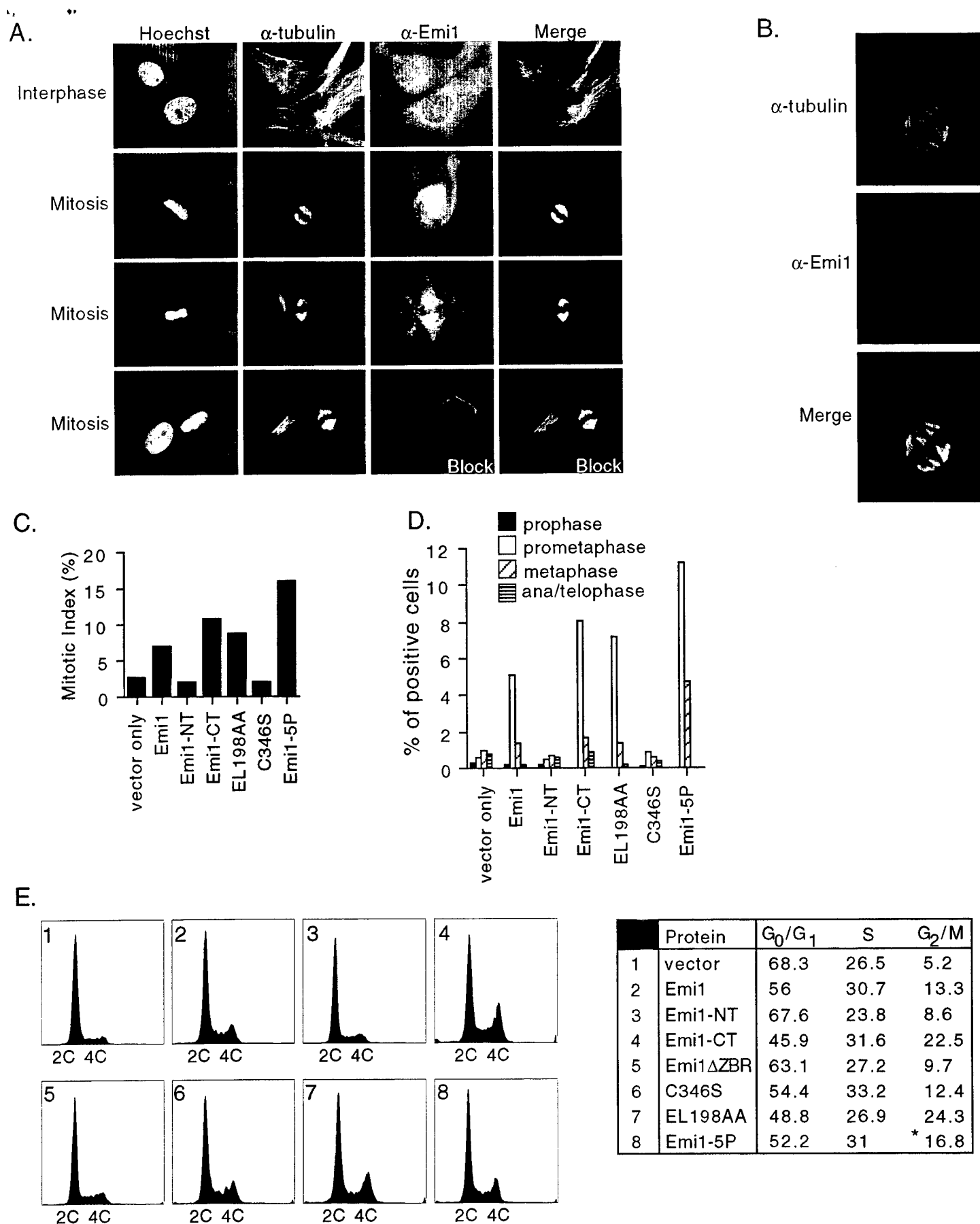
Reimann et. al. Figures 1B and 1C

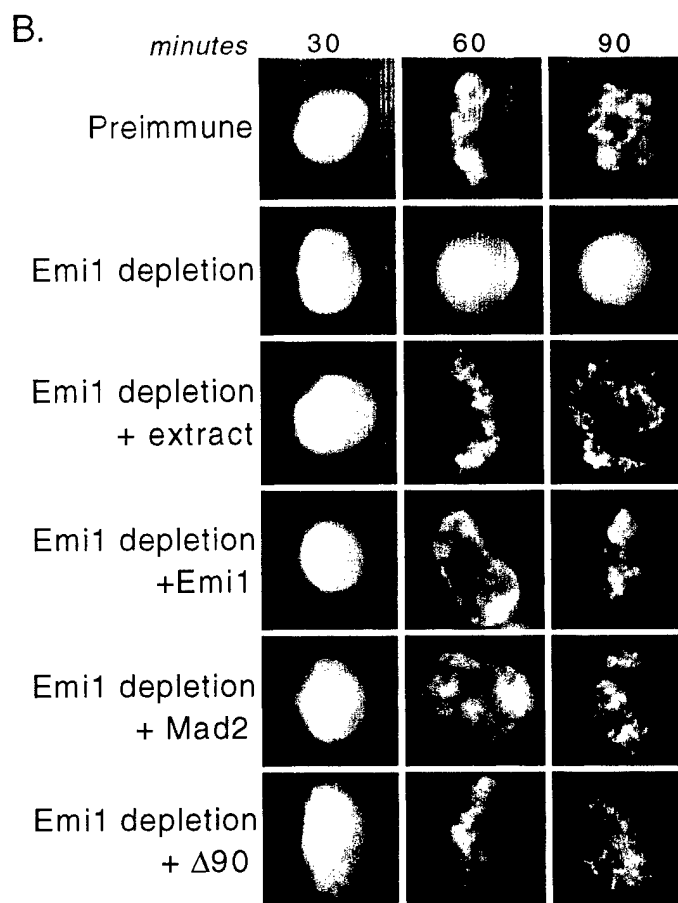
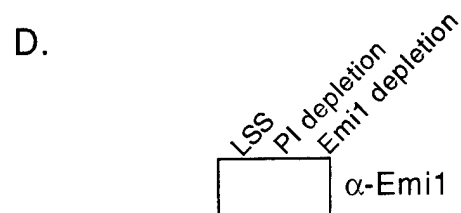
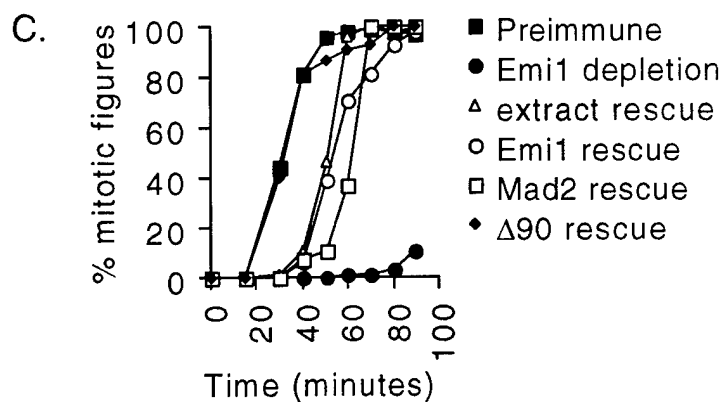
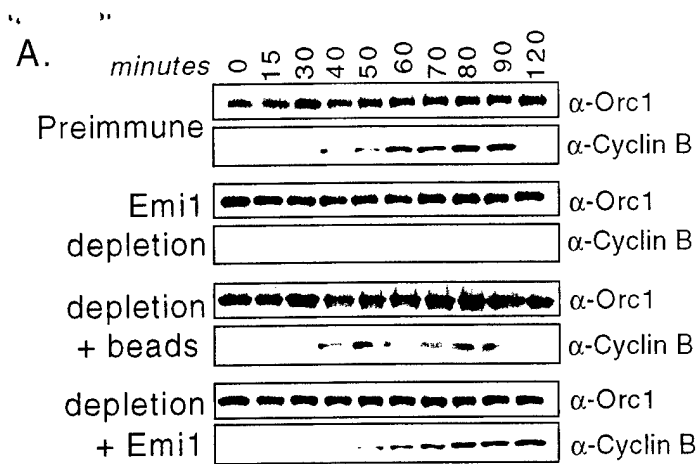


Flow cytometric analysis of the XTC cell cycle blocks for figure 2D.

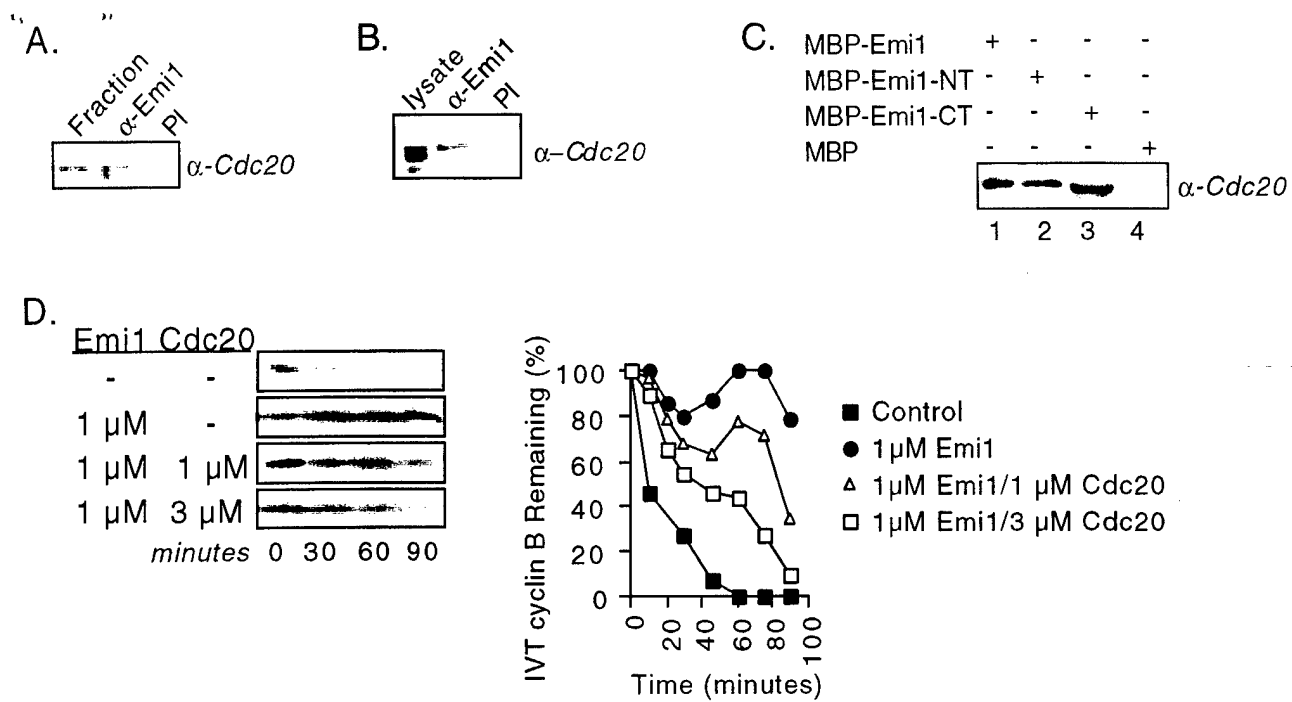




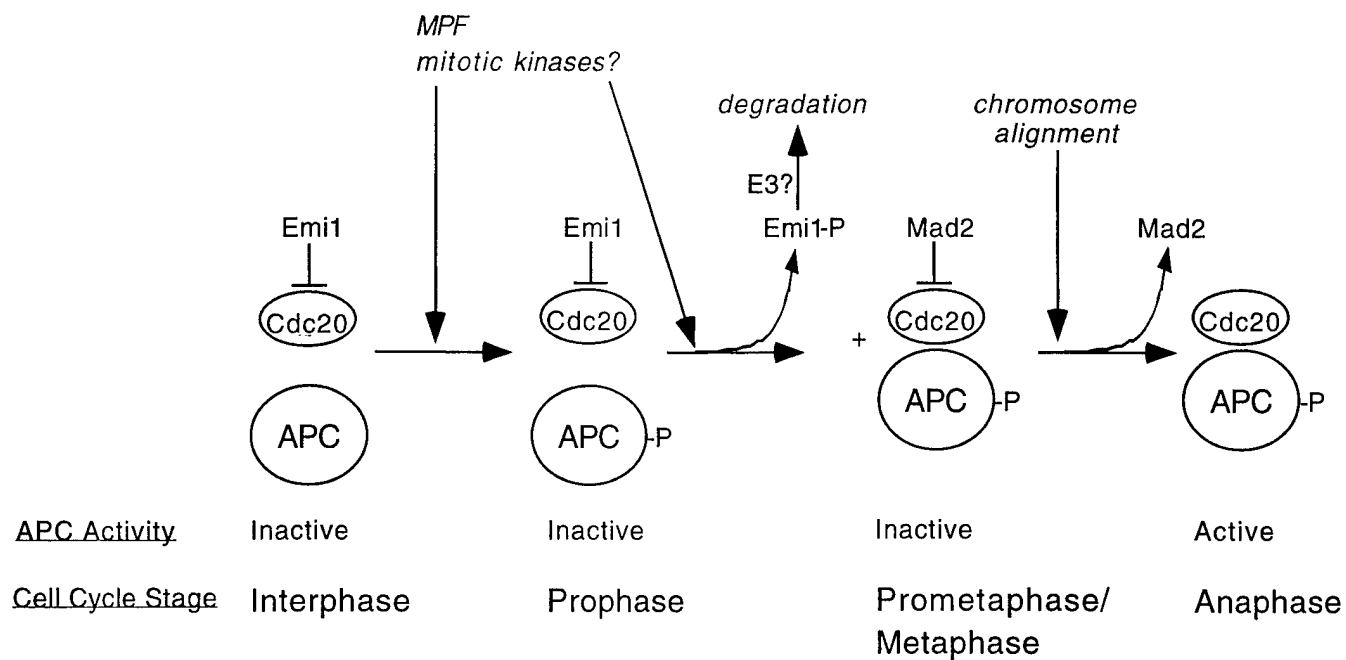




Reimann et. al. Figure 5



Reimann et. al., Figure 6



Reimann et. al., Figure 7

Triggering Cdk inhibitor ubiquitylation at origins of DNA replication

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A current model for triggering DNA replication in yeast and mammalian cells requires ubiquitin-mediated proteolysis of the cyclin-dependent kinase inhibitors that restrain the G1-S transition ^{1,2}. In mammals, the ubiquitylation of the cyclin E/Cdk2 inhibitor p27^{Kip1} requires its association with cyclin E/Cdk2 ³, phosphorylation of p27^{Kip1} on threonine 187 ⁴, and recognition of p27^{Kip1} by the SCF^{Skp2} ubiquitin ligase ^{3,5,6}. However, whether cyclin E/Cdk2 binding to p27^{Kip1}, the subsequent phosphorylation of p27^{Kip1}, the activity of the SCF^{Skp2} ubiquitin ligase, or other factor(s) control the timing of Cdk inhibitor destruction to initiate DNA replication remains unclear. We show here in egg and advanced gastrula embryo extracts from the amphibian *Xenopus* that ubiquitylation and destruction of the cyclin E/Cdk2 inhibitor p27^{Xic1} is independent of phosphorylation, but does require prior assembly of replication pre-initiation complexes containing ORC, Cdc6, and the MCM proteins. Further, Xic1 is recruited to these chromatin-associated complexes by cyclin E/Cdk2. Here, Xic1 is selectively ubiquitylated, ensuring that ubiquitylation and subsequent destruction of the Cdk inhibitor occurs only at the site of an activated replication origin. Therefore, the role that cyclin E/Cdk2 plays in timing ubiquitylation and origin activation is spatially constrained to the site of origin firing itself.

The requirement for ubiquitin-mediated proteolysis of a Cdk inhibitor to trigger DNA replication is conserved from yeast to humans. In budding yeast, ubiquitin-dependent destruction of Sic1, an inhibitor of the cyclin-dependent kinase that drives DNA replication, is critical for initiation of S phase ¹. The SCF^{Cdc4} ubiquitin ligase directs the ubiquitylation of Sic1 (reviewed in ⁷). Likewise, in mammals, the SCF^{Skp2} directs the ubiquitylation of the cyclin E/Cdk2 inhibitor p27^{Kip1}, which inhibits progression from G1 to S. The requirement for the F-box protein Skp2 in p27^{Kip1} instability has been shown in mice deficient for the Skp2 gene, and the *in vitro* competence of the SCF^{Skp2} complex for ubiquitylating p27 has likewise been demonstrated ^{3,5,6}. In human cells, the Cdk inhibitor p21^{Cip1} must also be degraded to allow S phase progression, although it is not clear whether the degradation of p21 requires ubiquitin ligases such as SCF^{Skp2} ⁸ or is independent of ubiquitylation ⁹. In *Xenopus* eggs, components of the SCF E3 ubiquitin ligase (PKJ unpublished) and its associated E2 ubiquitin conjugating

enzyme, Cdc34¹⁰, are required for DNA replication. Here, the specific target(s) of destruction are not known, although the Cdk2 inhibitor p27^{Xic1}, which is related to the human Cdk inhibitors p27^{Kip1} and p21^{Cip1}, is a good model^{11,12}.

Xic1 inhibits cyclin E/Cdk2 *in vitro*, and recent evidence supports that Xic1 accumulation helps determine the length of G1 at gastrulation¹³ and withdrawal from the mitotic cell cycle to promote neuronal differentiation^{14,15}. In egg extracts that cycle between S phase and M phase with virtually no intervening G1, Xic1 mRNA and protein levels are low¹¹. Nonetheless, the use of egg extracts to study the degradation of Xic1 has been previously validated^{10,16}. Further, our studies of Xic1 destruction in extracts prepared from mid to late blastula or early to mid gastrula embryos (Fig. 1D) are consistent with data from egg extracts. As in egg extracts, destruction of Xic1 in extracts made from stage 10-13 embryos is dependent upon SCF activity, Cdk2 activity, and the proteasome (not shown). In all *Xenopus* extract systems tested, nuclear accumulation of cyclin E/Cdk2 consistently triggers DNA replication and the nuclear-specific ubiquitylation and destruction of Xic1. Because Xic1 associates with cyclin E/Cdk2 in the nucleus, one hypothesis is that the nuclear accumulation of cyclin E/Cdk2 may primarily drive Xic1 phosphorylation and recognition by an SCF complex similar to Skp2.

To determine if Xic1 is destroyed through a phosphorylation-dependent mechanism, similar to p27^{Kip1}, we tested Xic1 phosphorylation site mutants in an assay for ubiquitylation and destruction coupled to nuclear transport (Fig. 1). We suspected that T205 of Xic1 would be required for Xic1 destruction because 1) T205 is within a region highly similar to the region containing the required phosphorylation site T187 in p27^{Kip1}, 2) *Xenopus* cyclin E/Cdk2 phosphorylates Xic1 primarily on T205 *in vitro*, and 3) this phosphorylation bypasses the nuclear requirement for Xic1 destruction¹⁶. Fig. 1A shows that the mutation T205A does not stabilize Xic1. Because there are six putative CDK phosphorylation sites in Xic1, we systematically mutated these sites, but found that no single point mutations or combinations of mutations stabilizes Xic1 (Fig. 1A). In fact, the sextuple mutant (Xic1-6A) which cannot be phosphorylated by cyclin E/Cdk2 *in vitro*, is destroyed at the same rate and to the same extent as the wild type

(Fig. 1A, compare first two rows). In these experiments, we controlled for the amount of IVT-Xic1 protein added. We also tested recombinant GST-Xic1 protein in this assay to determine if phosphorylation-site mutants are stabilized at higher concentrations than achieved with IVT product. However, even at 50 nM, the phosphorylation site mutants are degraded as readily as wild type Xic1 in terms of both kinetics (not shown) and extent (Fig. 1C).

This mutant analysis suggests that direct phosphorylation of Xic1 is not required for its destruction. However, inhibition of endogenous cyclin E/Cdk2 with the Cdk inhibitor p21 stabilizes the WT and Xic1-6A proteins. These data suggest that either p21 blocks the activity of cyclin E/Cdk2 to bind or phosphorylate other critical targets, i.e. replication factors, or p21 blocks the physical interaction of cyclin E/Cdk2 with Xic1. In addition, cyclin E/Cdk2 may also be required to activate components of the SCF itself.

We previously showed that Xic1 is ubiquitylated and destroyed within the nucleus, reproduced here (Fig. 2A). IVT-Xic1 is added to *Xenopus* extracts in the presence of sperm nuclei, and at the indicated times, the cytoplasmic and nuclear fractions are separated. Xic1 accumulates in the nuclear fraction after nuclei have formed (~40 min) and is rapidly ubiquitylated and destroyed there. Import of Xic1 into the nucleus depends on the import of its binding partner, the cyclin E/Cdk2 complex¹⁶. Our recent work demonstrates a requirement not only for nuclear accumulation of cyclin E/Cdk2, but also for its association with chromatin, to trigger initiation of DNA replication (LF, BKK, CS, and PKJ, manuscript submitted; Reviewer: see attached). To determine if Xic1 also accumulates on chromatin, we extracted the nuclear pellets with Chromatin Extraction Buffer (see Methods) and isolated the insoluble chromatin fraction (Fig. 2B). We have previously shown that this extraction procedure removes >90% of total nuclear protein, including complete extraction of known nucleoplasmic proteins (LF, BKK, CS, and PKJ, submitted). Fig. 2C shows that the majority of ubiquitylated Xic1 is in the chromatin fraction. Quantitation of these bands shows that on average the chromatin fraction contains >90% of total ubiquitylated Xic1. The kinetics of Xic1 accumulation on chromatin

parallels its accumulation within the nucleus (Fig 2B). Thus, Xic1 ubiquitylation occurs primarily on chromatin.

The bulk of cyclin E/Cdk2 is recruited to chromatin by a receptor composed of the Origin Recognition Complex (ORC) and the initiation factor Cdc6 (LF, BKK, CS, and PKJ, submitted). The mini-chromosome maintenance (MCM) proteins themselves require ORC and Cdc6, but not cyclin E/Cdk2, to be assembled onto chromatin ¹⁷ to perform their critical functions leading to replication initiation at origins. However, the presence of MCM proteins on chromatin is not required for cyclin E/Cdk2-chromatin recruitment (LF, BKK, CS, and PKJ, submitted). We have previously shown that cyclin E/Cdk2 is critical for the ubiquitylation and destruction of Xic1 ¹⁶. One function of cyclin E/Cdk2 is to facilitate the nuclear import of Xic1 ¹⁶. A second function of cyclin E/Cdk2 might be to direct Xic1 to chromatin. Depletion of cyclin E from extracts significantly reduced the amount of Xic1 bound to and ubiquitylated on chromatin (not shown). However, the directness of this effect is difficult to assess, because without cyclin E/Cdk2, Xic1 is not efficiently transported into the nucleus ¹⁶. Nonetheless, we have found that addition of excess cyclin E/Cdk2 to extract increases the amount of Xic1 recruited to chromatin (data not shown). If Xic1 recruitment to chromatin by cyclin E/Cdk2 is critical for its ubiquitylation, then the cyclin E/Cdk2 receptor proteins on chromatin, ORC and Cdc6, would also be required for Xic1 destruction. To test this, we immunodepleted ORC or Cdc6 from *Xenopus* egg extracts prior to assembling nuclei and isolating assembled chromatin. In parallel, we also depleted MCMs and assembled identical samples to determine whether a later step in chromatin assembly, beyond cyclin E recruitment, is required for SCF ubiquitylation activity towards Xic1.

We find that depletion of ORC (Fig. 3A) or Cdc6 (data not shown) stabilizes the Xic1 protein and prevents ubiquitylation of Xic1 on chromatin. Addition of immunopurified ORC or purified baculovirus expressed Cdc6 rescues Xic1 ubiquitylation in ORC- or Cdc6-depleted extracts. These results support the model that cyclin E/Cdk2 triggers Xic1 ubiquitylation by coupling Xic1 to pre-initiation complexes, rather than by phosphorylating Xic1. If the ORC/Cdc6 complex was sufficient to trigger Xic1 ubiquitylation, then depletion of the MCM

proteins, which are not required to recruit cyclin E/Cdk2 to chromatin, should not affect Xic1 ubiquitylation. To our surprise, depletion of MCMs inhibited Xic1 ubiquitylation (Fig. 3A), suggesting that after Xic1 is recruited to chromatin by cyclin E/Cdk2 binding to ORC-Cdc6, additional events downstream of MCM proteins trigger Xic1 ubiquitylation. These steps might include origin unwinding or the assembly of replication elongation complexes. We examined the ability of Xic1 to be ubiquitylated when nuclei with unwound origins and formed elongation complexes were stabilized with the polymerase α inhibitor, aphidicolin. We find Xic1 is efficiently ubiquitylated and destroyed in aphidicolin-treated extracts (Fig. 3B) suggesting that Xic1 ubiquitylation is triggered after MCM action, but before elongation ensues.

Together, these experiments support a model (Fig. 4) wherein the timing of replication origin firing is set by the nuclear accumulation of cyclin E and the activation of SCF-dependent proteolysis towards critical substrates including Cdk2 inhibitors like p27^{Xic1} in *Xenopus* and p27^{Kip1} in mammals. However, the ability of cyclin E to trigger SCF-activity and origin firing requires the association of cyclin E/Cdk2 and the recruitment of SCF substrates, including the Cdk inhibitors, to origin complexes on chromatin. Thus, the activation of cyclin E/Cdk2-triggered replication events is made dependent on the prior assembly of functional pre-initiation complexes. Although we do not know which component(s) of the assembled pre-initiation complex are responsible for activating the SCF towards Cdk inhibitors on chromatin, several candidates assemble downstream of MCMs in the chromatin assembly pathway. Recent work in *Xenopus* nucleoplasmic extracts has shown that chromatin must be exposed to active Cdc7/Dbf4 prior to exposure to Cdk2 activity to trigger efficient DNA replication¹⁸. A mechanism to ensure this order of events might include Cdc7 control of SCF activation towards Cdk inhibitors such as Xic1. Because Cdc7 activity is required to load another essential component of the preinitiation complex, Cdc45¹⁹, onto chromatin²⁰, Cdc45 is another possible candidate for controlling SCF activation to trigger destruction of the Cdk inhibitor and the activation of DNA replication.

In previous work, we showed that both cyclin E/Cdk2 and SCF components including Skp1 and Cull1 are localized to centrosomes, where they are critical for triggering centriole separation, an early step in the centrosome cycle ^{21,22}. Here we show yet another instance of SCF localization to an important site of cell cycle regulation, suggesting that localization of SCF ubiquitylation activity to critical sites of cell cycle transitions may facilitate their spatial regulation.

The mechanism by which Xic1 ubiquitylation relieves the inhibition of Cdk activity and leads to initiation remains unclear. Ubiquitylation of Xic1 may cause its direct proteolysis while still tethered to chromatin. Alternatively, the formation of a multi-ubiquitin chain on Xic1 at replication origins may serve a yet undiscovered function facilitating the unwinding of origins. Recent work from Reed and colleagues suggests that ubiquitylation of Met4 by the SCF^{Met30} complex causes the inactivation of a transcriptional regulator, independent of proteolysis ²³. Likewise, it is possible that Cdk inhibitor ubiquitylation may activate DNA replication independent of protein destruction.

Methods

Preparation of interphase, CSF, and mid-gastrula embryo extracts

Interphase ²⁴ and CSF ²⁵ extracts were prepared as described. We obtained identical results in chromatin association and destruction assays using either type of extract.

Developmental extracts were made by fertilizing laid *Xenopus* eggs and allowing progression to mid-gastrula stage. Embryos were supplemented with cytochalasin D (10 µg/ml) and spun as above. Opaque middle layer containing the majority of nuclei (40,000/µl) was diluted to 8,000 nuclei/µl and used for destruction assays (protocols available upon request).

Immunodepletion of extracts

Immunodepletions were performed by binding crude (XORC2, XMCM3) or affinity purified (XCdc6) rabbit antisera to protein A-Sepharose beads (O/N, 4 °C), incubating beads with extract (2 x 45 min, 4 °C), then separating beads from the extract by centrifugation in an Eppendorf microfuge (13,000 rpm, 10 min). Control depletions were performed with beads alone. Depletions were rescued with beads (XORC2 and MCM3) or purified recombinant protein (XCdc6).

Destruction assays

Destruction assays were performed as described ^{10,16}. Briefly, ³⁵S-labeled Xic1 (0.5 µl/10 µl extract), sperm (3000/µl), and an energy regenerating system were mixed with extract, incubated at room temperature, and stopped by dilution in sample buffer or centrifugation to separate cytosolic, nuclear, and chromatin fractions.

Nuclear and chromatin isolation assays

Nuclei were separated from the cytosolic fraction by sedimentation in a Beckman 152 microfuge (20 s) ¹⁶. Pelleted nuclei were resuspended in sample buffer and analyzed by Western blotting. Chromatin was extracted from a duplicate set of assembled nuclei by adding

10 volumes of Chromatin Extraction Buffer (50 mM KCl, 50 mM HEPES pH 7.7, 5mM MgCl₂, 5mM EGTA, 2mM β-mercaptoethanol, 0.5 mM spermidine, 0.15mM spermine, 0.1% NP-40), mixing gently, and leaving on ice for 30 min, prior to respinning the tubes as above (LF and PKJ, submitted). Protocols provided upon request.

Generation of Xic1 phosphorylation site mutants

Xic1 mutants of were created by PCR mutagenesis (Stratagene, Quickchange kit) and verified by sequencing. Primer sequences available upon request.

Production of recombinant Proteins

Different Xic1 proteins (³⁵S-labeled IVT-Xic1, MBP-Xic1, and GST-Xic1) behaved similarly in the assays described. ³⁵S-labeled Xic1 was prepared using *in vitro* coupled transcription/translation from the plasmid pCS2-Xic1¹⁰. GST- and MBP-Xic1 were purified from *E. coli* according to standard protocols.

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Figure Legends

Figure 1. Mutation of consensus CDK phosphorylation sites does not stabilize Xic1. A) Phosphorylation site mutants were generated, *in vitro* translated, and tested in the destruction assay as described in Methods. Serine and threonine were mutated to alanine (indicated by "A") in each mutant. Kinetic analysis of the wild type versus the sextuple phosphorylation site mutant (Xic1-6A) is shown (top two panels). Although all mutants were analyzed kinetically, only end point analysis for the other mutants is shown (bottom seven panels). B) A panel of phosphorylation site mutants were expressed and purified as GST fusion proteins and added to the destruction assay. Samples were resolved by SDS-PAGE and analyzed by western blotting with a polyclonal antibody raised against GST Xic1. C) p21 (100 nM) stabilizes the sextuple mutant Xic1-6A as well as the wild type. D) Soluble extracts made from mid-gastrula embryos were used to test the destruction of *in vitro* translated WT Xic1, Xic1 T205A, and Xic1-6A, as in Figure 1A.

Figure 2. Ubiquitylated forms of Xic1 accumulate on Chromatin. A) Xic1 is ubiquitylated and destroyed in the nucleus. Destruction assays using ^{35}S -labeled Xic1 were performed as described in Methods. Reactions were stopped at the indicated times and immediately separated into the cytoplasmic and nuclear fractions. One-fifth of the cytoplasmic and all of the nuclear fraction were resolved by SDS-PAGE and visualized by a phosphorimaging system. Subtypes of Xic1 are indicated: Xic1_{Cyt} represents the cytoplasmic fraction of Xic1, Xic1-Ub_{Nuc} represents the nuclear fraction of Xic1 that is not yet ubiquitylated, Xic1-Ub_n represents the nuclear fraction of Xic1 that is conjugated with ubiquitin molecules. B) The chromatin association of Xic1 mimics nuclear events. Intact nuclear pellets were washed in Chromatin Extraction Buffer. The insoluble chromatin-bound fraction was isolated by centrifugation as detailed in Methods. Samples were resolved and visualized as above. Xic1-Ub_{Chr} represents the chromatin fraction of Xic1 that is not yet ubiquitylated, Xic1-Ub_n represents the nuclear fraction of Xic1 that is conjugated with ubiquitin molecules. C) All of the nuclear

fraction is bound to chromatin. The nuclear (Nuc.) and chromatin (Chr.) fractions were compared by quantitation on the Phosphorimager and shown to be equivalent on average $\pm 10\%$.

Figure 3. Assembly of pre-replication complexes is required for Xic1 ubiquitylation. A) *Xenopus* egg extracts were mock depleted with beads alone or immunodepleted with beads conjugated to anti-ORC2 or anti-MCM3 antibodies. Depleted extracts were supplemented with sperm and ^{35}S -labeled IVT Xic1, destruction assays were performed, and chromatin fractions were isolated and visualized as in Fig. 2. Rescued samples were supplemented with the beads from the original immunodepletions. B) Samples were assembled and treated identical to 3A except that the proteasome inhibitor MG-132 (2 mM) was added to stabilize Xic1 in its various ubiquitylated forms (negating the need to take timepoints) and aphidicolin (40 $\mu\text{g}/\text{ml}$) was added to the indicated sample.

Figure 4. Model for the destruction of Xic1 at replication origins. Xic1 is recruited to origins of DNA replication by cyclin E/Cdk2, which directly associates with the pre-initiation protein Cdc6. Ubiquitylation of Xic1 depends on proper assembly not only of ORC and Cdc6, but also of the MCM complex, indicating that assembly of complete, functional origins sends a signal, here denoted as relaying through an unknown factor "X," to activate the ubiquitylation activity of SCF^{Skp2} towards p27^{Xic1} . Likely candidates for "X" include Cdc7/Dbf4 and Cdc45.

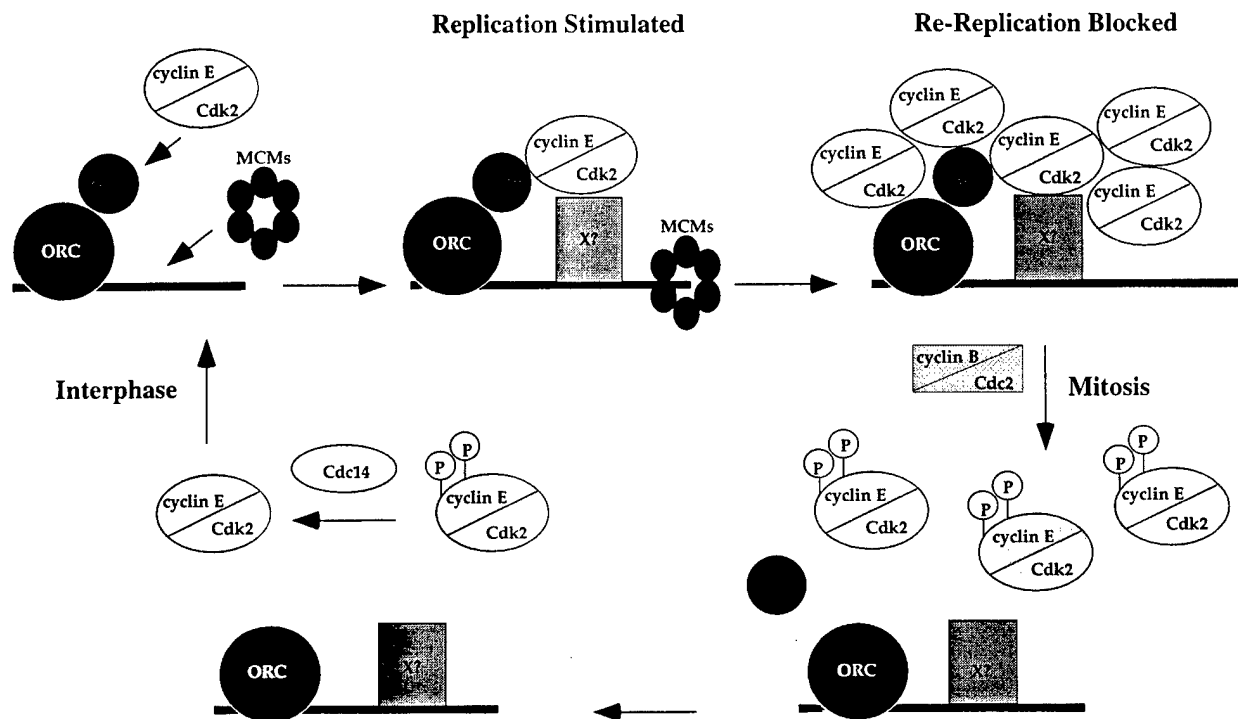
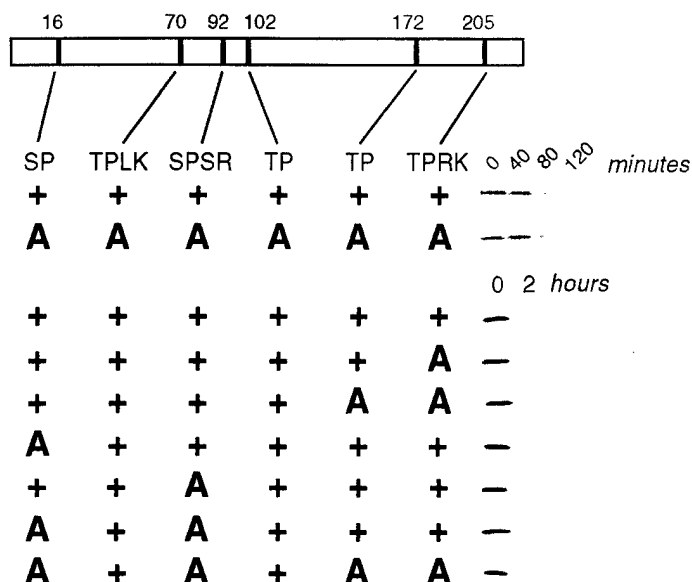


Figure 10, Furstenthal et al.

A.

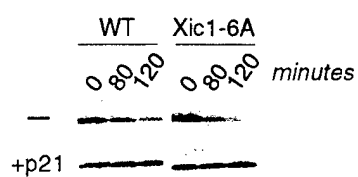


B.

S16	S92	T172	T205	0	2	hours
+	+	+	+	+	+	
A	A	A	A	+	+	
A	A	+	+	+	+	
+	+	A	A	+	+	

αXic1

C.



D.

